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**Advancing tendon healing by scaffolds-free delivery of
different subtypes of mesenchymal stem or progenitor cells**

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Abbreviations

bFGF	basic fibroblast growth factor
bHLH	basis helix-loop-helix
BM	bone marrow
ECM	extracellular matrix
ED	empty defect
ESC	embryonic stem cells
GDF	growth/differentiation factor
hAT	human Achilles tendon
hMSC	human mesenchymal stem cells
IGF	insulin-like growth factor
iPS cells	induced pluripotent stem cells
NT	native tendon
PAS	periodic acid schiff
PDGF	platelet-derived growth factor
PDL	periodontal ligament
S.O	safranin O
Scx	Scleraxis
TGF- β	transforming growth factor- β
Tnmd	tenomodulin
TSPC	tendon-derived stem and progenitor cells
VEGF	vascular endothelial growth factor

Publication list

Publication 1

Scaffold-free Scleraxis-programmed tendon progenitors aid in significantly enhanced repair of full-size Achilles tendon rupture

Chi-Fen Hsieh, Paolo Alberton, Eva Loffredo-Verde, Elias Volkmer, Matthias Pietschmann, Peter Müller, Matthias Schieker and Denitsa Docheva

Nanomedicine (Lond). 2016, 11(9):1153-67

Publication 2

Periodontal ligament cells as alternative source for cell-based therapy of tendon injuries: in vivo study of full-size Achilles tendon defect in a rat model

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European Cells and Materials. 2016, 32: 228-240

1. Introduction

1.1 Tendon injury and healing process

Tendons are dense connective tissues connecting muscle to bone, they transfer muscle forces onto bones and joints. For this function, tendons are composed of hierarchically organized collagen type I fibers providing the tendons' ability to withstand great tensile forces. Tendon injuries may result in joint dysfunction because they oftentimes heal by production of an inferior tendon matrix and are prone to re-occur. Tendon injuries are frequent medical problems in Traumatology and Orthopedics and mainly associate with overuse activity and age-related degeneration. Degenerative tendinopathy is considered one of the leading causes for tendon rupture and it also can result in unsuccessful healing response and increase risk of re-rupture (Yang *et al.*, 2013). Degenerative tendinopathy (tendinosis) can be characterized by different tissue alterations and certain forms are enriched in cartilage nodules, disorganized extracellular matrix, ectopic bone, hypervascularity or mucoid deposits (Riley, 2008). Tendons are poorly vascularized tissues with a low density of specialized mesenchymal cells which exhibit low mitotic activities (Benjamin and Ralphs, 1997). Overall, tendons have a slow turnover of their cellular and extracellular matrix content, therefore, tendon natural healing process is extremely low and inefficient especially in the elderly (Marsolais and Frenette, 2005). Calcifying tendinosis is described by calcium deposits within tendon proper and/or insertions, which can furthermore exert inflammation, pain, swelling, redness and limited motion (Rui *et al.*, 2011).

Tendon healing includes three consecutive stages: inflammation, proliferation and remodeling (Fig. 1) (Docheva *et al.*, 2015). The remodeling stage includes two sub-stages: consolidation and maturation. This stage is time consuming and is characterized by a decrease in cellular density, significant cell shape flattening, improved cell alignment to the axis of mechanical stretch, which is further accompanied by maturation of collagen type I and elastic fibers. Additional features of this stage are decreasing of biglycan and increasing of decorin and lumican protein expression (Juneja and Veillette, 2013). In parallel, collagen and elastic fibers start to organize along the longitudinal axis of the tendon, thereby restoring tendon stiffness and tensile strength (Sharma and Maffulli, 2005). The whole healing process after injury can go up to 1–2 years depending on the age and condition of the patient (Docheva *et al.*, 2015). Moreover, the number of tendon clinical interventions per year is constantly increasing, which is in a positive relation to the rising sports participation and aging population in USA and Europe (Butler *et al.*, 2004; Hess, 2010; Lantto *et al.*, 2015). Certain tendons (Achilles, rotator cuff, patella and forearm extensors) are particularly susceptible to degeneration and surgical treatment (Docheva *et al.*, 2015). Until now, the standard therapeutic options used for tendon repair comprise of surgical suture, cryotherapy, physiotherapy (including massage and controlled motion), shock-wave therapy, eccentric exercise therapy, autografts, allografts or synthetic prostheses; however, none of these alternatives has provided a

successful long-term solution and full regain of the tendon tissue quality and function particularly in aged individuals (Hsieh *et al.*, 2016a).

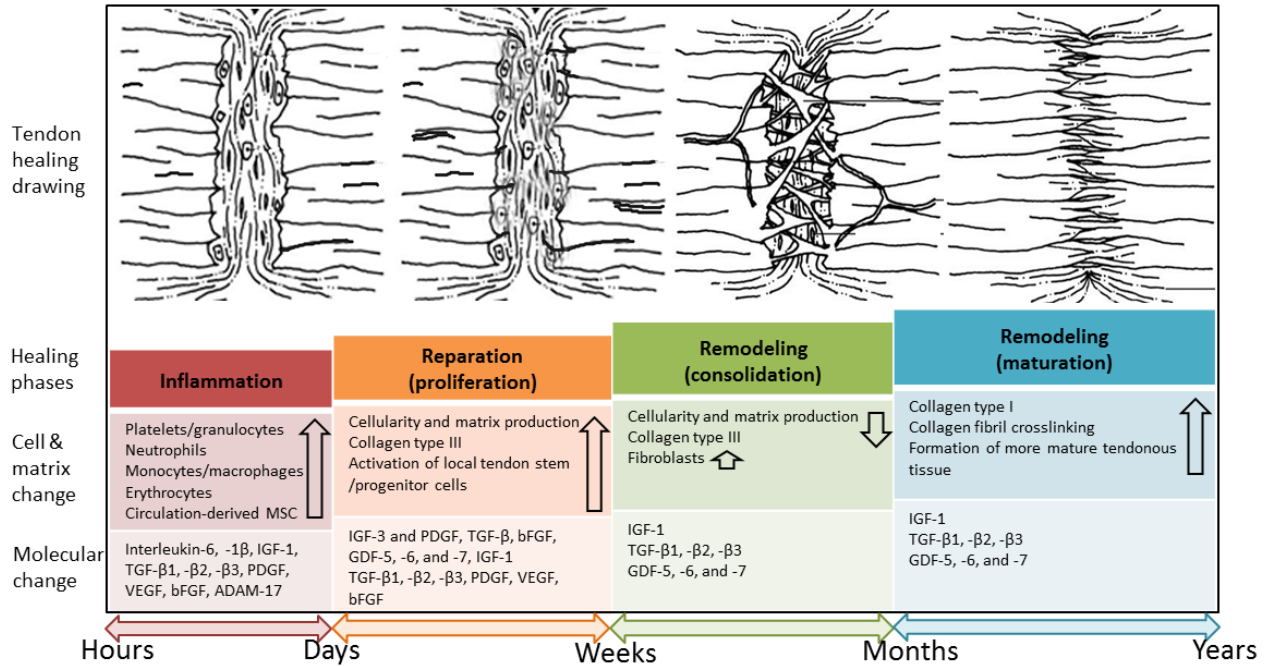


Fig. 1: The tendon repair process in humans. Four cartoon images corresponding to the main steps of tendon healing. Key cellular, matrix and molecular changes occurring in this process are described and graphically annotated. Based on (Beredjikian, 2003) and (Docheva *et al.*, 2015).

1.2 Tendon tissue engineering

The poor repair capacity of tendon requires novel strategies to speed up the healing process and to maximize the functional outcome. The successful healing of tendon injuries depends on various factors, including anatomical position, patient aged and physical condition, blood vessel supply, the depth and width of the wound and the amount of tissue loss. Although spontaneous healing can occur, this often results in scar tissue formation which is morphologically, biochemically and biomechanically different and inferior to healthy tendon tissue. These ultimately influence the functionality of the repaired tissue (Galvez *et al.*, 2014; Yang *et al.*, 2013). Hence, new and prospective developments in tissue engineering become increasingly important. The field of tissue engineering is highly multidisciplinary and spans from cell biology, molecular biology, medicine, and material science to mechanical engineering. In principle tendon tissue engineering relies on reparative cells, alone or in combination with biocompatible scaffolds, which are delivered during patient's surgery into the site of tissue damage (Hsieh *et al.*, 2016a). For the clinical need further research in the stage of pre-clinical development and optimization is needed (Fig. 2) (Docheva *et al.*, 2015). Therefore, selecting the most appropriate cell type is one of the essential factors to be considered in cell-based tissue engineering.

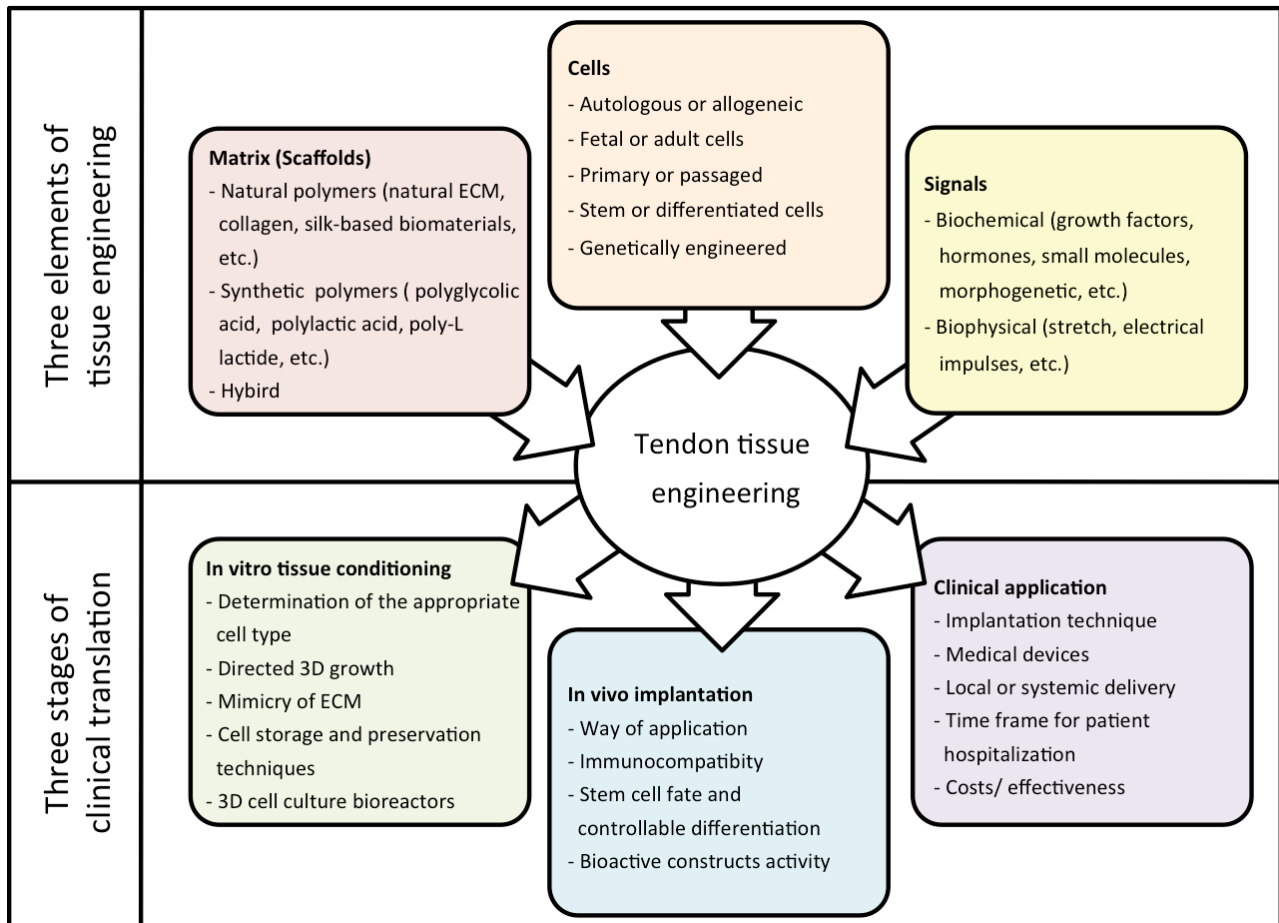


Fig. 2: The current strategies for tendon tissue engineering, future challenges and translation to clinics. Matrix, cells and signals are the three major elements of tissue engineering approaches to tendon repair. In vitro tissue conditioning, in vivo implantation and clinical application are three research parts of clinical translation for tendon tissue engineering.

1.3 Stem cell sources

Stem cells come from a variety of sources including embryonic stem cells (ESC), induced pluripotent stem cells (iPS cells) or adult stem cells (Fig. 3). ESC presented in the early embryo has one main disadvantage that is tendency to form teratomas. IPS cells are formed by co-expression of certain genes from adult somatic differentiated cells (Takahashi and Yamanaka, 2006). Using iPS cells avoids the ethical matters, but at present the technology is inefficient in generating large number of cell and not yet fully developed for clinical trials as well as there are still some concerns about tumor formation in vivo. Therefore, adult stem cells from autograft tissue are another more suitable and attractive cell type circumventing the ethical issues of ESC. The optimal cell type has to be able to adhere to the defect, differentiate to tissue-specific cells or exert stimulatory functions and trophic support to local progenitors. In addition, the function of the reparative cells can be enhances by growth factors or by genetic modification. Among the adult stem cell the best know so far are the one deriving from bone marrow. Concerning the use of bone marrow mesenchymal cells (BM-MSC) in tendon tissue engineering, one

advantage is that they can be obtained easier than tendon-derived stem/progenitor cells; via bone marrow aspirate from the iliac crest or femoral head. Furthermore, BM-MSC are well characterized and largely studied in various in vivo models. However, when used for repair of tendon tissue BM-MSCs, without tenogenic pre-differentiation, frequently lead to ossification, thereby worsening the tendon healing (Docheva *et al.*, 2015). Regarding tendon -derived cells application of autologous cell will avoid immune complications but the patient has to undergo extra surgery to obtain a tendon biopsy for purification of tendon cells causing comorbidity, therefore such use is not foreseeable in clinics. Application of allogeneic cells may be easier to obtain from cadavers but may lead to immune reactions. Furthermore, tendon-derived cells undergo a phenotypic drift during in vitro prolonged cultivation (Kohler *et al.*, 2013; Schulze-Tanzil *et al.*, 2004; Yao *et al.*, 2006). Bi *et al.*, 2007 (Bi *et al.*, 2007) were the first to compare the performance of BM-MSC to tendon-derived stem and progenitor cells (TSPC). This study showed that when the cells were applied in vivo, BM-MSC and TSPC behaved differently. TSPC formed tendon-like structures whereas BM-MSC formed bone-like structures. However, in this study, the cells were not tested in a clinically relevant tendon defect model.

Dental stem cells have been discovered in the last years, such as postnatal dental pulp stem cells, periodontal ligament stem cells, tooth germ progenitor cells, gingival MSCs, etc. (Liu *et al.*, 2015). These dental tissue-derived MSCs not only display self-renewal and multipotential but also possess immunomodulatory functions and potent tissue regenerative properties (Hakki *et al.*, 2015; Lei *et al.*, 2014; Liu *et al.*, 2015). Recently, several groups, including our, reported that periodontal ligament contains endogenous stem/progenitor cells (PDL) (Bluteau *et al.*, 2008; Docheva *et al.*, 2010; Ivanovski *et al.*, 2006). PDL are simply harvested from extracted teeth when patients have orthodontic treatments (Miura *et al.*, 2003; Seo *et al.*, 2004).

In order to investigate the most suitable adult stem cell types for tendon tissue engineering, we have established the isolation and cultivation of stem/progenitor cells from Achilles tendon (hAT cells) (Kohler *et al.*, 2013) and periodontal ligament (PDL cells) (Docheva *et al.*, 2010). These cells showed typical stem cell characteristics: they were positive for MSC surface markers (CD105, CD90 and CD73) and were able to differentiate into adipogenic, chondrogenic and osteogenic lineages. In contrast to the BM-MSC, the hAT PDL cells expressed Scleraxis (Scx) and tenomodulin (Tnmd), genes that are typical tenogenic markers (Dex *et al.*, 2016)

Genetically modifying MSCs prior to implantation may decrease the risk of ectopic bone formation as well as improve the performance of the cells. For example, the overexpression of the so far known tendon transcription factors, namely early growth response protein 1, Scx, and Mohawk in MSC have been reported to by us and others to lead to tenogenic differentiation prior to delivery (Guerquin *et al.*, 2013; Alberton *et al.*, 2012; Gulotta *et al.*, 2011; Liu *et al.*, 2015). In 2012, we genetically modified BM-MSC via lentiviral transduction of Scx cDNA (hMSC-Scx cells). We demonstrated that Scx overexpression was

successfully achieved and second, it resulted in reduction of self-renewal potential, loss of osteogenic and chondrogenic differentiation potential, and upregulation of *Tnmd* and other tendon-related genes. Thus, we concluded that the hMSC-Scx cells differentiated in tendon/ligament progenitors (Alberton *et al.*, 2012).

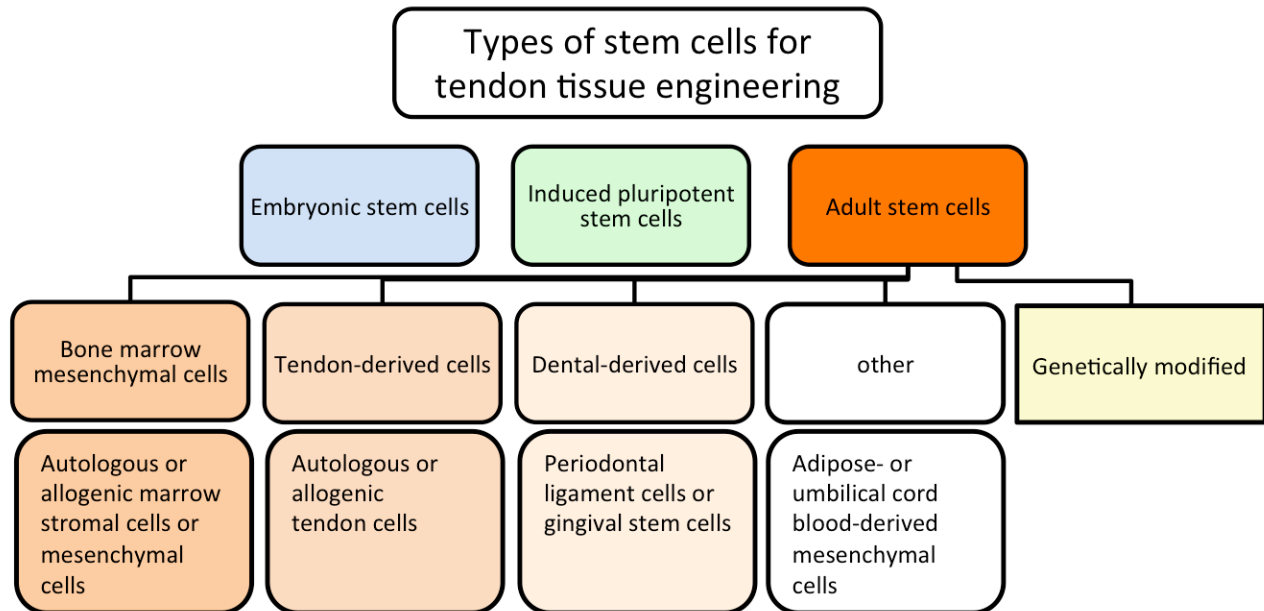


Fig. 3: Candidate stem cells types for tendon tissue engineering.

1.4 Objectives of the study

As discussed in the introduction due to the global population aging, age-related degeneration, traumatic sport injuries and the weak repair potential of tendons, tendon conditions still present major challenges to orthopedic medicine. A novel approach to treat tendon injuries is with tissue engineering, in which reparative cells are introduced into the defect in order to enhance the repair and to fully restore the original tissue strength and function. In this approach a critical step is to identify the most appropriate cell types. Previously we have (Pietschmann *et al.*, 2013) established a clinically relevant full-size Achilles tendon defect model in *Rattus norvegicus*. Therefore, the main aims of the doctoral thesis study were to investigate the potential of two novel and promising candidates' namely (1) Scleraxis-programmed tendon progenitors (hMSC-Scx cells) and (2) periodontal ligament-derived cells (PDL cells) in our *in vivo* model and to evaluate the effects of these two cell types on tendon repair in the critical late remodeling phase.

1.4.1 Publication 1

Scaffold-free Scleraxis-programmed tendon progenitors aid in significantly enhanced repair of full-size Achilles tendon rupture

In the recent years the interest towards tendon development and repair mechanisms has greatly increased. During embryonic development, tendon and ligaments, in the axial skeleton, descend from a sub-

compartment of the sclerotome called syndetome. The tendon progenitors, which are defined by the expression of the transcription factor Scx, derive from uncommitted mesenchymal progenitors, (Alberton *et al.*, 2012). Scx is a member of the basic helix-loop-helix (bHLH) transcription factor family to which belong muscle key transcription factors MyoD and Myogenin. At embryonic day 15.5, Scx transcripts become more restricted to tendons and ligaments and remain such during adulthood (Asou *et al.*, 2002). Moreover, Murchison *et al.*, 2007 (Murchison *et al.*, 2007), have shown that gene knockout of Scx in *Mus musculus*, leads to a severe tendon and ligament phenotype. Furthermore, Scx is a tensile force-inducible gene in association with PDL fibrogenesis and maturation under tensile stress transmitted from physiological and orthodontic conditions (Takimoto *et al.*, 2015). Thus, confirming the indispensable role of Scx for tendon and ligament development and function.

Hence, in our study we aimed to fully explore the potential of a scaffold-free delivery of hMSC-Scx cells in contributing to tendon repair by utilizing our clinical full-size defect model in rat Achilles tendons. (Pietschmann *et al.*, 2013) and carrying out in-depth histo-morphometric examinations after 16 weeks of cell implantation. We selected this time point in order to assess the long-term effect of the cells and how advanced is the critical late remodeling phase of tendon healing (Hsieh *et al.*, 2016a).

We have already established a tendon rupture model in a Foxn1nu athymic rat strain (*Rattus norvegicus*) (Pietschmann *et al.*, 2013). The model consisted of a surgical removal of three millimeter tissue from the Achilles tendon proper. The defects were reconnected by Kirchmayr–Kessler suture and it was filled by a three dimensional cell pellet of 1×10^6 cells (Fig.1A of publication 1). Four different study groups were generated (with ten animals per group): group (1) – included non-injured tendon (native tendon, NT); group (2) – control group consisting of animals with empty defect (ED); groups (3) and (4) – in which the animals received non- (hMSC-Mock) or Scx-modified (hMSC-Scx) cell pellets, respectively. In each animal only one hind limb was subjected to operation and 16 weeks were given for repair. The whole calcaneus-Achilles-gastrocnemius complexes of the rats were obtained from all groups and were used for various molecular and histological analyses.

First, the gross morphological appearances and point-based scoring system showed that hMSC-Scx group had smaller mean tendon width and superior composition of the healed Achilles tendons. Next, by performing nuclear morphology and cell density studies, we concluded that tendons, which received our engineered cells after injury contain more tendon-like cells pointing towards an early maturation and advance healing. Third, the X-ray images and bone-related protein marker analysis revealed that the hMSC-Scx group has the smallest ossification area when compared to the ED and hMSC-mock groups. Then, periodic acid schiff (PAS) and Safranin O (S.O) staining demonstrated that the hMSC-Scx group has decreased ectopic fibrocartilage formation and very low proportion of mucopolysaccharides within the defect site. Lastly, the results of polarized light microscopy and immunohistological analyses indicated that hMSC-Scx group has higher collagen I, decorin, lumican, elastin levels and advanced matrix

maturation, hence making this study group the closest to the NT. In addition, using human-specific gene PCR and immunohistochemical staining for human antigen, we also confirmed the existence, localization and integration of the human cells into the injured tissue even after 16 weeks, suggesting their direct contribution to the healing. Taken together, we concluded that hMSC-Scx cells carry out powerful tendon inducing and differentiating properties and their implantation results in faster and better tendon repair; thus, making them very suitable for reconstruction of full-size tendon defects.

In future, in order for hMSC-Scx cells, or variants of this technology, to become a factual clinical tool in orthopedics several open questions have to be addressed, such as possible combination and optimization with biomaterials/carriers; understanding the exact fate and survival rate of implanted cells; examining stable programming via transient Scx expression in autologous and allogenic cells; long-term functional and biomechanical performance tests in big animal models without immunosuppression. We believe that the present and forthcoming results on the application of Scx-based cell therapy can lead to improve outcomes of tendon healing (Hsieh *et al.*, 2016a).

1.4.2 Publication 2

Periodontal ligament cells as alternative source for cell-based therapy of tendon injuries: in vivo study of full-size Achilles tendon defect in a rat model

Periodontal ligament is a novel attractive cell source because it can be obtained from exfoliated deciduous teeth of children aged 5 onwards and from sound teeth that are normally discarded in frequent routine orthodontic procedures (premolars in class II malocclusion or wisdom teeth removal) in dental clinics. Patient-specific PDL cells can be easily harvested by scratching the surface of the root and *in vitro* pre-expanded to high numbers in simple standard culture conditions. Possible bacterial, fungal or mycoplasma contamination can be easily controlled by using antibiotics, antifungal agents or elimination kits. Harvested PDL cells can thereafter be stored up for years ahead and last the phenotype of the cells is very similar in nature to tendon cells, hence no complicated genetic modification or growth factor manipulations are required. Therefore, the PDL can be considered an ideal source for stem cell banking and be used in therapy of tendon injuries (Hsieh *et al.*, 2016b).

Here, the same Achilles tendon defect model as publication 1 and similar experimental group design have been used with four study groups: group (1) –non-injured tendon (native tendon, NT); group (2) –empty defect tendon (ED); groups (3) and (4) –with PDL or hAT cell pellet implantation, respectively.

First, macroscopic observation and H&E staining presented that PDL and hAT groups have smaller mean width and larger tendinous areas with well-organized collagen fibers. Second, PAS and S.O staining demonstrated that the remnant cartilaginous areas within the defect site are almost resorbed in PDL and hAT groups. Next, antibody-based stainings for collagen type I and III, polarized light microscopy and ELISA assays for collagen type I proved that the repaired tendons of PDL group had similar collagen

organization to hAT group and significantly better than ED group. Third, the results of immunohistological stainings (biglycan, decorin, lumican and elastin) and ELISA assays for biglycan and lumican demonstrated that the repaired tendons of PDL group had comparable matrix maturation to hAT group and advanced one to ED. Lastly, the results of cell density and angular deviation investigations showed close cell morphometry PDL and hAT groups to NT. Moreover, the implanted PDL and hAT cells were detected by human-specific immunohistochemical staining suggesting direct contribution as well as long-term survival in the defect area. Taken together, we concluded that the PDL cells aid in tendon healing in very similar fashion to hAT cells and therefore, can be considered as attractive alternative source for cell-based therapy of tendon injuries. Several open questions remain to be clarified in following investigations such as possible combination of PDL cells with suitable biomaterials; the scaling up to dimensions similar to human tendon injuries; the reached biomechanical strength; the risk of PDL cell quality loss after long-term storage; the exact technique and time point of application; and the prognosis on periods for patient hospitalization and post-operative physiotherapy. It will be of great interest, by using adequate animal models, to examine wheatear PDL-derived cells are also suitable for prevention or therapy of tendon/ligament degenerative diseases, that are occurring prior rupture, and for periodontitis. We trust that further research on these questions will be critical to translating our animal model to potential clinical applications (Hsieh *et al.*, 2016b).

2. Summary

Introduction: Current treatment options of injured tendons mostly include surgical interventions, tendon transfer or conservative therapy; however, frequently they do not provide satisfactory results and the repair process is very lengthy. Moreover, the patient's quality of life is majorly affected and injury relapses may occur. Cell-based tendon tissue engineering is attractive alternative approach to speed up and augment tendon repair. Choosing the most appropriate cell type is essential in this application as it can greatly affect the success of the therapy. At present, there are various obstacles of using certain subsets of adult stem cells such as mesenchymal stem cells (MSC) from bone marrow (BM) and tendon-derived cells in tendon engineering. We have previously established and characterized mesenchymal stem cells, which are genetically modified with tendon-specific transcription factor Scleraxis (hMSC-Scx) as well as patient-derived periodontal ligament cells (PDL). Here, the main goal was to evaluate the repair potential of these cell types in a clinically relevant rat full-size Achilles tendon defect and to evaluate their benefits on tendon repair at the critical remodeling stage of the tendon healing process.

Materials & Methods: Cells were pre-expanded using standard culture conditions and pelleted prior implantation in scaffold-free fashion. Four study groups were design for the evaluation of each cell type (controls: native tendon (NT, non-injured Achilles tendon); empty defect (ED, consisting of injured Achilles tendon without cell implantation); hMSC-Mock (implanted with cells expressing mock transgene); hAT (implanted with Achilles tendon-derived cell line) and full-size tendon injury model was applied as described by us in (Pietschmann *et al.*, 2013). In brief, the model consists of: 1) a surgical removal of three millimeters tissue from the Achilles tendon proper; 2) reconnection of the tendon ends by Kirchmayr–Kessler suture; 3) administration of cell pellet; 4) post-implantation pellet adherence for 5 min; and 5) skin closure. The Achilles tendons were harvested for detail histological and molecular evaluation at 16 weeks after surgery.

Results: Evaluating hMSC-Scx healing potential. Firstly, quantitation of the Achilles tendons dimensions was used to assess the gross morphological appearances in four experimental groups. We found that the hMSC-Scx group had significantly smaller mean tendon width compared with the ED group. Next, we applied H&E and elastin staining for point-based scoring system. Our scoring data clearly showed that the hMSC-Scx group had a superior composition of the healed Achilles tendons that is closer to the NT group. We investigated the cellular alignments, cell flattening and cell density by H&E- and DAPI-stained images and the quantification results showed that the hMSC-Scx group had better nuclear orientation angles, lower cellular density and higher abundance of flattened cells compared to the ED and hMSC-Mock groups. Additionally, using x-ray and osteopontin stained images; we demonstrated that the hMSC-Scx group had the least degree of calcification in comparison to the ED and hMSC-Mock groups. Finally, based on the combination of various histological (periodic acid schiff and Safranin O staining), polarized

light microscopy and immunohistological analyses of critical tendon matrix proteins (type I, III collagen, biglycan, decorin, lumican and elastin), the hMSC-Scx group demonstrated superior repair, more organized collagen fibers and advanced matrix maturation of the injured tendons, making this study group the closest to the native non-injured tendon. Via two different techniques for in vivo tracking, namely forensic genomic PCR and antibody-based detection, we could demonstrate that the human cells are retained within the implanted area and that a portion of these cells or their progeny can survive as long as 16 weeks post-surgery. Thus, our hMSC-Scx cells not only can form tendon-like structures, but can directly contribute to enhanced tendon healing in a clinically relevant Achilles tendon injury model.

Evaluating PDL healing potential. The gross appearance and H&E-stained mosaic images, PDL-implanted Achilles tendons suggested that this group performs comparably to the hAT group, but also is superior to the non-implanted injured tendon group, ED. PDL groups exhibited smaller mean tendon width compared to the ED group. Next, matrix histology, bone marker osteopontin staining and quantitation of X-ray images revealed that the implantation of either PDL or hAT cells was advantageous for the tendon healing process. Based on various immunohistological analyses (type I, III collagen, biglycan, decorin, lumican and elastin), polarized light microscopy and ELISA quantitation (type I, III collagen, biglycan and lumican), we concluded that the PDL group, similar to the hAT group, has more mature and organized matrix protein composition than the ED group. Last, we evaluated the cell density and cellular alignment as described above and our results showed that the PDL group had lower cell density and better parallel alignment than the ED group. Furthermore, the implanted human cells or their progeny were detectable by human-specific antigen HLA-ABC, thus suggesting long-term integration in the tendon. In sum, PDL cells can become very attractive novel cell source for tendon and ligament therapy due to their easier collection from orthodontic procedure and banking up for later use in clinics.

Conclusion: Our in-depth results demonstrated that hMSC-Scx and PDL cells implantation in injured tendons leads to superior tissue maturation, more organized collagen fibres and better tissue/cell morphometric parameters when compared with the non-implanted tendons. Taken together, the outcomes suggest that hMSC-Scx and PDL cells exert strong tendon inductive and repair properties making them well suited for reconstruction of tendon defects.

3. Zusammenfassung

Einleitung: Aktuelle Behandlungsmöglichkeiten von verletzten Sehnen umfassen vor allem chirurgische Eingriffe, Sehnenübertragung oder konservative Therapie; jedoch liefern häufig diese Eingriffe kein zufriedenstellendes Ergebnis und der Reparaturprozess ist sehr langwierig. Darüber hinaus ist die Lebensqualität des Patienten stark beeinträchtigt und Verletzungen können wiederauftreten. Zell-basierte Sehnen-Tissue-Engineering ist eine attraktive Ansatzalternative zur Beschleunigung und Verbesserung von Sehnenreparaturen. Die Wahl des am besten geeigneten Zelltypen ist von wesentlicher Bedeutung zur Anwendung, da es großen Einfluss auf den Erfolg der Therapie ausübt. Derzeit gibt es verschiedene Hindernisse bei der Verwendung bestimmter Stammzellpopulationen adulter Stammzellen, wie mesenchymale Stammzellen (MSC) aus dem Knochenmark (BM) und Sehnen abgeleiteten Zellen in der Sehnen-Tissue-Engineering. Wir haben zuvor mesenchymale Stammzellen etabliert und charakterisiert, die mit dem Sehnen-spezifischen Transkriptionsfaktor Scleraxis (hMSC-Scx) modifiziert sind wie auch parodontale Ligamentzellen (PDL). Hier war das Hauptziel, das Reparaturpotenzial dieser Zelltypen in einem klinisch relevanten Achillessehnendefekt der Ratte zu beurteilen und ihre Vorteile bei der Sehnenreparatur im kritischen Remodelierungsstadium des Sehnenheilungsprozesses zu bewerten.

Materialien & Methoden: Die Zellen wurden unter Standardkulturbedingungen vorexpanziert und vor der Implantation in gerüstoffreier Weise pelletiert. Es wurden vier Studiengruppen für die Beurteilung der einzelnen Zelltypen (Kontrollen: native Sehne (NT, nicht verletzte Achillessehne), leerer Defekt (ED, bestehend aus verletzter Achillessehne ohne Zellimplantation: hMSC-Mock (implantiert mit Zellen)) und das Sehnenverletzungsmodell in voller Größe angewendet wie bereits beschrieben in (Pietschmann *et al.*, 2013). Kurzgefasst, das Modell besteht aus: 1) einer chirurgischen Entnahme von drei Millimeter Gewebe von der eigentlichen Achillessehne; 2) Wiederverbindung der Sehnenenden durch Kirchmayr-Kessler-Naht; 3) Verabreichung von Zellpellets; 4) Post-Implantation eine Pellet-Adhärenz für 5 min; und 5) Hautverschluss. Die Achillessehnen wurden zur detaillierten histologischen und molekularen Auswertung nach 16 Wochen nach der Operation entnommen.

Resultate: Auswertung des Heilpotenzials der hMSC-Scx. Zunächst wurde die Quantifizierung der Achillessehnenmaße verwendet, um die groben morphologischen Erscheinungen der vier experimentellen Gruppen zu beurteilen. Wir stellten fest, dass die hMSC-Scx-Gruppe eine signifikant kleinere mittlere Sehnenbreite aufwies als die ED-Gruppe. Als nächstes verwendeten wir H&E und Elastin-Färbung für ein Punkt-basierendes Bewertungssystem. Unsere Bewertungsdaten beweisen deutlich, dass die hMSC-Scx-Gruppe eine überlegene Zusammensetzung der geheilten Achillessehnen nachweist, die näher an der NT-Gruppe ist. Wir untersuchten die zellulären Ausrichtungen, die Zellabflachung und Zelldichte durch H&E- und DAPI-gefärbte Bilder. Die Quantifizierungsergebnisse zeigten, dass die hMSC-Scx-Gruppe

bessere Kernorientierungswinkel, geringere Zelldichte und höhere Häufigkeit von abgeflachten Zellen im Vergleich zu den ED- und hMSC-Mock-Gruppe nachwies. Anhand von Röntgen- und Osteopontin gefärbten Bildern bewiesen wir dass die hMSC-Scx-Gruppe den geringsten Grad der Verkalkung im Vergleich zu den ED- und hMSC-Mock-Gruppen aufweist. Abschließend, basierend auf die Kombination verschiedener histologischer (Periodsäure-Schiff- und Safranin-O-Färbung), polarisierter Lichtmikroskopie und immunhistologischer Analysen kritische Sehnenmatrixproteine (Collagen Typ I und III, Biglycan, Decorin, Lumican und Elastin), zeigte die hMSC-Scx Gruppe deutlich überlegene Reparatur, mehr organisierte Kollagenfasern und erweiterte Matrix-Reifung der verletzten Sehnen, somit zeigte sich diese Studiengruppe der nativen, nicht verletzten Sehne am nächsten. Durch zwei verschiedene Techniken für die in vivo-Verfolgung, nämlich forensische genomische PCR und den Antikörper-basierten Nachweis, konnten wir belegen, dass die menschlichen Zellen innerhalb des implantierten Bereichs zurückgehalten werden und dass ein Teil dieser Zellen, oder deren Nachkommen, bis zu 16 Wochen nach dem chirurgischen Eingriff überleben können. So können unsere hMSC-Scx-Zellen nicht nur sehnenartige Strukturen bilden, sondern auch direkt zu einer verbesserten Sehnenheilung in einem klinisch relevanten Achillessehnenverletzungsmodell beitragen.

Auswertung des Heilspotenzials von PDL. Anhand des gesamten Aussehens und H&E-gefärbte Mosaikbilder, deuten PDL-implantierte Achillessehnen darauf hin, dass diese Gruppe vergleichbar mit der hAT-Gruppe ist, aber auch der nicht implantierten verletzten Sehnengruppe ED überlegen ist. PDL-Gruppen zeigten eine kleinere mittlere Sehnenbreite im Vergleich zur ED-Gruppe. Als nächstes zeigten Matrixhistologie, Knochenmarker-Osteopontin-Färbung und Quantifizierung von Röntgenbildern, dass die Implantation von PDL- oder hAT-Zellen für den Sehnenheilungsprozess vorteilhaft war. Basierend auf verschiedenen immunhistologischen Analysen (Kollagen Typ I und III, Biglycan, Decorin, Lumican und Elastin), der polarisierten Lichtmikroskopie und der ELISA-Quantifizierung (Kollagen Typ I und III, Biglycan und Lumican) wurde festgestellt, dass die PDL-Gruppe, ähnlich der hAT Gruppe, mehr reife und organisierte Matrix-Protein-Zusammensetzung als die ED-Gruppe nachwies. Zuletzt haben wir die Zelldichte und die zelluläre Ausrichtung wie oben beschrieben evaluiert. Unsere Ergebnisse zeigten, dass die PDL-Gruppe eine niedrigere Zelldichte und eine bessere parallele Ausrichtung als die ED-Gruppe aufweist. Darüber hinaus konnten die implantierten menschlichen Zellen, oder ihre Nachkommen, durch human-spezifisches Antigen HLA-ABC nachgewiesen werden, was auf eine Langzeitintegration in die Sehne hindeutet. Zusammengefasst, können PDL-Zellen als eine sehr attraktive neue Zellquelle für Sehnen- und Ligamentherapie fungieren aufgrund ihrer einfacheren Sammlung von kieferorthopädischen Verfahren und Banken für die spätere Verwendung in Kliniken.

Schlussfolgerung: Unsere eingehenden Ergebnisse zeigten, dass hMSC-Scx- und PDL-Zellimplantationen in verletzten Sehnen zu einer überlegenen Gewebereifung, mehr organisierter Kollagenfasern und besserer morphometrischer Gewebe/Zellen Parameter führt im Vergleich zu den nicht implantierten Sehnen.

Zusammengenommen zeigen die Ergebnisse, dass hMSC-Scx und PDL-Zellen starke Sehnen induktive und Reparatur-Eigenschaften ausüben, wodurch sie gut für die Rekonstruktion von Sehnendefekten geeignet sind.

4. Publication 1

Scaffold-free Scleraxis-programmed tendon progenitors aid in significantly enhanced repair of full-size Achilles tendon rupture

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Scaffold-free Scleraxis-programmed tendon progenitors aid in significantly enhanced repair of full-size Achilles tendon rupture

Aim: Currently there is no effective approach to enhance tendon repair, hence we aimed to identify a suitable cell source for tendon engineering utilizing an established clinically relevant animal model for tendon injury. **Materials & methods:** We compared, by in-depth histomorphometric evaluation, the regenerative potential of uncommitted human mesenchymal stem cells (hMSC) and *Scleraxis* (*Scx*)-programmed tendon progenitors (hMSC-Scx) in the healing of a full-size of rat Achilles tendon defect. **Results:** Our analyses clearly demonstrated that implantation of hMSC-Scx, in contrast to hMSC and empty defect, results in smaller diameters, negligible ectopic calcification and advanced cellular organization and matrix maturation in the injured tendons. **Conclusion:** Scaffold-free delivery of hMSC-Scx aids in enhanced repair in a clinically translatable Achilles tendon injury model.

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Keywords: mesenchymal stem cells • *Scleraxis* • tendon healing • tendon progenitors

Tendons injuries, consecutive to overuse or age-related degeneration, are common and present a clinical challenge in orthopedic medicine because of the lengthy periods of post-traumatic healing and functional gain, as well as the high rate of rupture recurrence [1,2]. Moreover, the number of tendon clinical interventions per year is constantly increasing, which is in direct relation to the rising population aging in Europe and the USA [3–5]. Certain tendons (Achilles, rotator cuff, patella and forearm extensors) are particularly susceptible to degeneration and surgical treatment. The standard therapeutic options for tendons repair comprise of suture, autografts, allografts and synthetic prostheses; however, none of these alternatives have provided a successful long-term solution, especially in aged individuals [6].

Tendon tissue heals by initial formation of poor scar tissue which is then gradually remodeled over long periods of time [7–12]. In short, the healing involves three overlapping

phases: an inflammatory stage, which occurs immediately after the tendon rupture; a proliferation stage begins within the first days/week and results in the establishment of scar tissue reconnecting the injured tendon ends; and a long remodeling stage including the two substages; consolidation and maturation, initiating around 6 weeks after injury and goes up to 1–2 years depending on the age and condition of the patient [7–12]. Cellularity and matrix production are gradually decreased during the consolidation stage followed by gradual increase of collagen fibril alignment and cross-linking over the extended period of the maturation phase [7–12]. And precisely because tendon repair and rehabilitation requires lengthy periods, the field's great interest is to accelerate these processes without loss of quality and function the *de novo* built tissue. Cell-based tissue engineering is nowadays one of the most attractive and widely explored strategies for musculoskeletal regeneration. This approach

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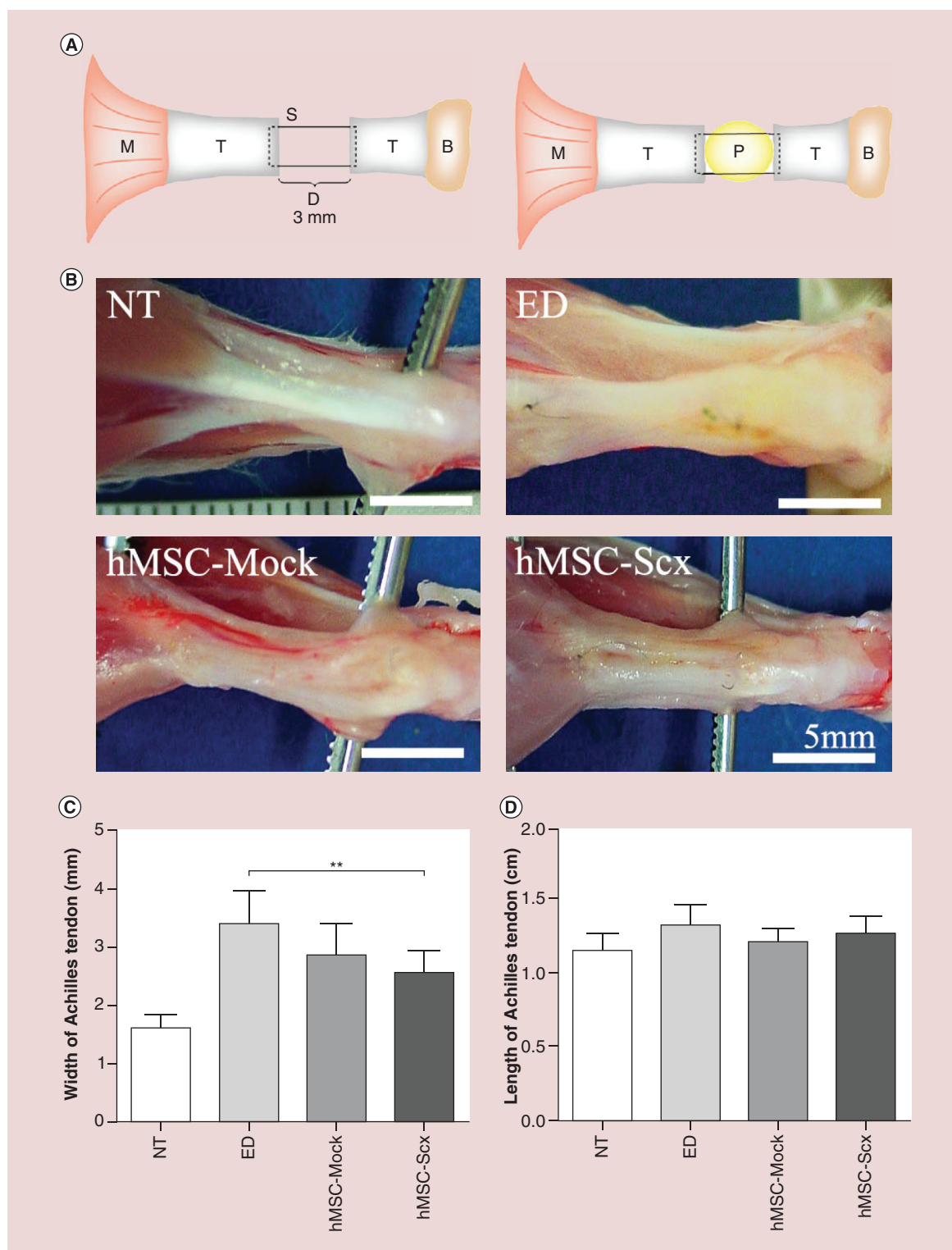


Figure 1. Investigation of Achilles tendon overall appearance and dimensions. (A) A schematic image of the established Achilles tendon injury model. (B) Representative gross morphological images of the four study groups, NT, ED, hMSC-Mock and hMSC-Scx after 16 weeks of healing. (C) The width, measured in the tendon proper, and (D) length of the Achilles tendons from the four groups. Bar chart present mean values \pm standard deviations.

** $p = 0.002$ hMSC-Scx vs ED ($n = 10$, 10 animals/group).

B: Calcaneus bone; D: Surgery defect; M: Gastrocnemius muscle; P: Cell pellet; S: Surgical suture; T: Achilles tendon.

relies on reparative cells, alone or in combination with biocompatible scaffolds, which are delivered during patient's surgery into the site of tissue damage. Therefore, selecting the most appropriate cell type is of utmost importance.

In tendon engineering, several cell types such as mesenchymal stem cells (MSC) from different tissue sources and tendon-derived cells have been suggested as suitable targets. However, the involvement of each of these cell types has to solve various challenges (reviewed in [7]). Bone marrow (BM)-MSC are the most frequently evaluated cell type due to well-established collection protocols as well as being the best characterized cells; however, ectopic bone formation within the injured tendons is closely associated with BM-MSC implantation (reviewed in [7]). The main risks of autologous tendon cell applications are patient comorbidity and a phenotypic drift of the primary cells during the steps of *in vitro* expansion and validation ([13,14], and reviewed in [7]).

Scleraxis (Scx) is a member of the basic helix-loop-helix (bHLH) transcription factor family, which regulates embryonic tendon formation and postnatal tendon function [15,16]. In 2012, we showed that a stable ectopic expression of *Scx* in BM-hMSC resulted in the generation of a homogeneous population of committed tendon progenitors [17]. In 2013 [18], we published the establishment of a very clinically relevant full-size Achilles tendon injury model in *Rattus norvegicus*. Recently, three studies, namely by Gulotta *et al.*, Chen *et al.* and Tan *et al.*, have tested whether *Scx* in combination with cells and carriers can promote early tendon repair up in animal models [19–21]. Hence, in our study we aimed to fully explore the potential of a scaffold-free delivery of hMSC-*Scx* cells in contributing to tendon repair by utilizing the above *in vivo* model [18] and carrying out in-depth histo-morphometric examinations after 16 weeks of cell implantation. We selected this time point in order to assess the long-term effect of the cells and how advanced the critical late remodeling phase of tendon healing is.

Materials & methods

Cell culture

The well-established and characterized hMSC-Mock and hMSC-*Scx* cell lines were used in the study (described in [17]). These two cell lines were maintained in Alpha minimum essential medium GlutaMAX culture media (Gibco, Germany) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Germany). Cells were cultured on polystyrene dishes in a humidified incubator at 5% CO₂ and 37°C. Cells were expanded to sufficient for implantation cell numbers in three-five consecutive passages. Prior

implantation, aliquots of hMSC-Mock and hMSC-*Scx* were used for RNA isolation and polymerase chain reaction (PCR) validation of transgene expression (Supplementary Figure 1 & Supplementary data), which has been previously demonstrated in [17].

Animal model & surgical procedure

Surgical procedures were performed according to our established *in vivo* model with slight modifications [18]. All procedures for animal handling prior, during and after surgery were approved by the Animal Care and Use Committee of the Bavarian Government (animal grant no. 55.2-1-54-2531-58-08). Briefly, athymic female Foxn1nu rats were purchased from Charles River (Sulzfeld, Germany) and accommodated in the central animal facility. Four different study groups with ten rats per group were randomly selected from the colony: (1) Native tendon (NT), consisting of noninjured left Achilles tendon; (2) Empty defect (ED) group, consisting of injured right Achilles tendon without cell implantation; (3) and (4) in which the animals received one-shot implantation of scaffold-free Mock- (hMSC-Mock group) or *Scx*-hMSC pellets (hMSC-*Scx* group) within injured right Achilles tendon, respectively. Cell centrifugation at 500 g for 10 min was carried out to form a 3D cell pellet comprising of 1×10^6 cells. The surgical operation consisted of surgically removing three millimeters tendon tissue from the Achilles tendon proper (~3 mm proximal from the calcaneus and 3 mm distal from the gastrocnemius muscle, average length of rat Achilles tendon is 12 mm) (Figure 1A). Each defect was first reconnected by Kirchmayr–Kessler suture and then the 3D cell pellet was administered with a cut sterile pipette tip and left to adhere for approximately 5 min (Figure 1A).

At 16 weeks after surgery animals were euthanased, gastrocnemius muscle – Achilles tendon – calcaneus bone units revealed by skin incision, imaged and width of tendon proper and total length of the Achilles tendons were manually measured. Afterward tissue samples were explanted and processed as described below.

Histomorphometry

The gastrocnemius muscle – Achilles tendon – calcaneus bone units were fixed either in 4% paraformaldehyde (PFA; Merck, Germany) or in 95% ethanol/5% absolute acetic acid overnight at 4°C (each five animals/group). PFA-fixed sections were used for general histological and immunohistochemical stainings, while ethanol-fixed sections were used for immunohistochemistry with anticollagen antibodies (Supplementary Table 1). After fixation, specimens were decalcified in 10% EDTA/PBS pH 8.0 (Sigma-Aldrich, Germany) for four weeks at room tempera-

Point-based scoring system for histological and elastic fiber assessment in order to evaluate the progress of Achilles tendon repair.

Study group	Scored parameters							VII Total points
	I Cellularity	II Ectopic calcification	III Vascularized areas	IV Adipose areas	V Poor fibrous tissue	VI Elastic fibers		
						Content	Morphology	
NT								
Mean	4	4	3.5	2.95	4	4	4	26.45
Median	4	4	3.5	2.95	4	4	4	26.45
IQR	0	0	1	1.3	0	0	0	
ED								
Mean	1	2.7	3.17	2.73	1.77	2.25	1.25	14.87
Median	1	3	3	2.6	1.65	2.5	1	14.75
IQR	0	1.1	0.25	0.55	1.475	2.25	0.75	
hMSC-Mock								
Mean	1.24	2.72	2.76	2.5	1.5	2.5	1.75	14.97
Median	1.6	3	3	2.6	1.3	2.5	1.5	15.5
IQR	1.3	1.3	1.2	0.65	1.1	1	1.75	
hMSC-Scx								
Mean	1.5	3.33	2.8	2.9	2.48	2.75	2.25	18.01
Median	1	3.15	2.8	3	2.65	3	2.5	18.1
IQR	1.5	0.825	1.6	0.3	2.78	0.75	1	

Mean, median point values and IQR (interquartile range) of scored points are shown.
I: Estimation of increase in cellularity compared with normal, uninjured tendon (NT).
II: Estimation of increase in areas with ectopic calcification compared with NT.
III: Estimation of increase in vascularized areas compared with NT.
IV: Estimation of increase in adipose-rich areas compared with NT.
V: Estimation of increase in areas with poor fiber organization compared with NT.
VI: Estimation of increase in total amount of mature elastic fibers compared with NT.
VII: The sum of points of classes I–VI.
4 points = 0–25%, 3 points = 26–50%, 2 points = 51–75% and 1 point = 76–100%. For elastin fiber morphology 4 points = thin parallel elastin fibers, while 1 point = thick irregular elastin fibers.

ture, then treated with consecutive 10, 15 and 20% sucrose/PBS solutions at 4°C for 2 h and last, embedded in tissue freezing medium (Jung, Germany), and cryosectioned longitudinally at 12 µm (Microm HM500 OM, Germany) allowing full exposure of the whole complex Gastrocnemius muscle- Achilles tendon-calcaneus. Prior use sections were stored at -20°C.

Selected sections (PFA-fixed) were stained with hematoxylin and eosin (H&E) [18], or periodic acid-schiff (PAS) using well-established standard histological protocols or following manufacturer’s instructions (PAS staining system, Sigma-Aldrich). For the Safranin-O/Fast Green (S.O/F.G) staining [18,22], the sections were equilibrated to room temperature and hydrated with PBS 3× 5 min and then changed to deionized water. Next, the sections were placed in 0.02% F.G (Sigma-Aldrich) in 95% ethanol for 12 min, rinsed under 1% Acetic Acid for 5 min, immersed

in 0.5% S.O (Sigma-Aldrich) for 15 min. Last, samples were rinsed quickly in 90% and 100% ethanol, and mounted with Entellan mounting medium (Merck).

For immunohistochemistry, selected sections were treated with 2 mg/ml hyaluronidase (Sigma-Aldrich, Germany) for 30 min at 37°C. After washing and blocking, primary antibodies were applied overnight at 4°C. Next day, corresponding secondary antibodies were used for 1 h. For full details on blocking solutions and antibodies used in the study refer to [Supplementary Table 1](#). For immunofluorescence-based experiments, sections were shortly counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Life technology, USA) and mounted with fluoroshield (Sigma-Aldrich, Germany). For 3,3' diaminobenzidine (DAB)-based experiments, an initial quenching step was performed using 4% H₂O₂/methanol for 30 min at room temperature, and afterward the classical

vectastain ABC kit was implemented according to the manufacturer's instructions (VectorLab, USA). Photomicrographs were taken with a black/white Axiocam MRm or color AxiocamICc3 camera mounted on Observer Z1 microscope (Carl Zeiss, Germany). Individual staining types were reproduced three-times independently and for each studied parameter according to the initial fixation, three to five animals per group were analyzed.

In order to evaluate the progress of tendon repair, H&E- or consecutive elastin-stained sections from five animals (each animal represented with three to five different slides consisting of three to four sections) were employed for microscopical scoring by four independent observers in a blinded fashion. A point-based scoring system for histological and elastic fiber assessment was used similar to Stoll *et al.* [23] with some modifications. The following parameters were scored and given points: (I) percentage of increase in cellularity compared with NT; (II) percentage of increase in ectopic calcification; (III) percentage of vascularized areas versus total area; (IV) percentage of adipose-like-occupied areas; (V) percentage of poor fibrous areas; (VI) elastin fiber content (percentage increase) and morphology; and (VII) total score as the sum of points in parameters I-VI (Table 1).

Quantification of nuclear aspect ratio, orientation & density

In healthy tendon or in advanced stage of tendon healing, low numbers of cells with much flattened nuclei that are parallel-aligned to the tensile axis are typical features. To determine the aspect ratio of nuclei (NAR, defined as the ratio of the minor diameter to the maximal diameter) and cell density via nuclear count, DAPI images were analyzed using ImageJ software v1.45s with installed NII plugin (National Institutes of Health, MD, USA). First, by color threshold adjustment the images were converted in black and white wherein DAPI-stained areas appear in black color and the remaining background in white color. Second, by implementing 'analyze particles' tool in NII plugin nuclear numbers and minor and maximal diameter for each nucleus were automatically calculated. Third, NAR were next calculated with GraphPad software and distributed by frequency as flattened cells are represented by $NAR = 0$, while rounded cells have $NAR = 1$. Cell density was expressed by calculating the number of nuclei per mm^2 .

To determine nuclear orientation (by estimating angular deviation), H&E-stained images were analyzed with the following algorithm using the Axio-Vision V4.8 software (Carl Zeiss): first, each image was converted to grey scale and nuclei were marked

by adjusting the intensity segmentation threshold; second, nuclear orientation measurements were made with the 'angular' tool as the angle between the major axis of a nucleus and the longitudinal axis of the tissue were automatically given for manually selected nuclei. Nuclear orientation angle values of 0° represent a nucleus that is perfectly aligned along the longitudinal axis and as this value increases toward 90° , the nucleus becomes more angled to the long axis of the tendon.

Six to nine randomly taken $20\times$ magnification images per section (within the area of injury 3 mm proximal from the calcaneus) of three to five animals per group were analyzed and a total number of approximately 2000 nuclei were measured for NAR and density, while approximately 200 nuclei per group were evaluated for angular deviation.

X-ray analysis

Dissected hind limbs from the four study groups (ten animals/group) were screened for in-tendinous calcification using x-ray imaging with a Cabinet x-ray Faxitron (Hewlett Packard, USA). Images were taken with 32 kV, 2 mA and 2 s exposure time. White areas comparable with the calcaneus represented calcified areas. The total area of calcification in each animal was digitally quantified in each Achilles tendon using the 'polygonal' tool of Image Pro Plus software v4 (Media Cybernetics, USA). In addition, mean value and standard deviations were calculated for each study group.

Polarized microscopic evaluation

Organized collagen type I fibers have strong birefringence property when exposed to polarized light; hence, they can be visualized in orange to yellow colors using microscopy. All 10 animals per group were prescreened and four representative animals/group were selected for the best quality of the H&E slides allowing for optimal and identical setting of the polarized microscopy imaging. Each animal was represented with one slide consisting of three sections. Microscopy was performed with $10\times$ objective supplemented with a polarized filter mounted on Axioskope 2 microscope (Carl Zeiss). For optimal imaging the transmission axis of the analyzer was with an angle of 54° to the axis of the polarizer in the analyzes of all samples. In order to cover the entire tendon territory approximately 50 consecutive images/section were taken manually in a mosaic manner and digitally stitched with Adobe Photoshop CS5 software (Adobe System, CA, USA). Next, total Achilles tendon area (denominator) was measured in pixel with 'magic wand' tool and by using 'color selecting' tool the yellow-to-orange-positive pixels (numerator), corresponding to highly organized collagen areas, were automatically quantified. Final data were expressed

as a percentage of collagen birefringence and for each study group mean values and standard deviations were calculated.

Detection of the implanted human cells in rat Achilles tendons

For detection of the implanted hMSC-Mock or hMSC-Scx, tissue sections were stained with human-specific (HLA)-ABC antibody as described above and in [Supplementary Table 1](#). For the detection of human DNA in rat tendons genomic PCR-based method was used as follows: the material of five tissue sections from 10 animals/group was scraped, collected and lysed with 20 mg/ml proteinase K in PCR buffer (Merck) overnight at 55°C. For controlling sample loading PCR for rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH, primers 5'-GACATGCCGCTGGAGAAAC-3' and 5'-AGCCAGGATGCCCTTTAGT-3') was performed using the following PCR program: 95°C/5 min; 95°C/30 s; 56°C/30 s; 72°C/60 s; 72°C/1 min; as steps 2–4 38 cycles. PCR with human specific genomic primers for amelogenin X (AMELX, primers 5'-CAGCTTCCCAGTTTAAGCTCT-3' and 5'-TCTCCTATACCACTTAGTCACT-3' [24]) was performed with touchdown program: 94°C/5 min; 94°C/30 s; 57°C/30 s; 4. 72°C/30 s; 94°C/30 s; 47°C/30 s; 72°C/30 s; 72°C/5 min as steps 2–4 10 cycles and steps 5–7 38 cycles. MGRsearch PCR instrument (BioRad, Germany) was used. The PCR products were separated on 2% agarose gels, stained with ethidium bromide and visualized on a gel imager (Vilber Lourmat, Germany).

Data & statistics analysis

Quantitative data and statistical significance were analyzed with GraphPad Prism 5 software (GraphPad, CA, USA). In general, each group was segregated in two halves due to fixation procedures; then three to five animals per group were examined, but the total histology slide and section numbers were kept equal between groups. In width, length, scoring and x-ray analyses all ten animals were evaluated. Bar charts show mean values and standard deviations. Multigroup statistical testing was performed with one-way ANOVA followed by *post hoc* Dunnett's test and t-test, and p-values < 0.05 were considered statistically significant.

Results

Engineered Achilles tendons with Scleraxis-programmed tendon progenitors are superior in tissue appearance & composition

The gross morphological appearances of the Achilles tendons of the four experimental groups were evaluated and documented post-dissection. We concluded

that all tendon defect ends, including the ED group, were completely reconnected. Among the three injured groups, the healed Achilles tendons of the hMSC-Scx group had the best opalescent tissue appearance that resembled the native tissue ([Figure 1B](#)). According to our quantitative data, the hMSC-Scx group had significantly smaller mean tendon width compared with the ED group ([Figure 1C](#)), whereas no significant differences were found regarding the mean tendon length ([Figure 1D](#)).

For examination of the general tendon composition, organization as well as for overall assessment of the healing stage first, H&E ([Figure 2A](#)) and elastin stainings and imaging of the four groups were performed. Afterward a qualitative evaluation of the degree of repair in all injured Achilles tendons was carried out by point-based scoring of the following parameters ([Supplementary Figure 2](#)): cellularity, ectopic calcification, vascularized areas, adipose areas, poor fibrous areas and elastic fiber content and morphology. The results of four independent blinded observers are given in [Table 1](#). Our scoring data clearly showed that the hMSC-Scx group (total mean of 18.01 and median of 18.1) has the highest scores compared with the other injured groups (ED group: total mean of 14.87 and median of 14.75; hMSC-Mock group: total mean of 14.97 and median of 15.5), indicating a superior composition of the healed Achilles tendons that is closer to the noninjured, healthy tendon NT group.

Very flattened cell nuclei, parallel alignment of the cells along the longitudinal strain axis and low cellularity are characteristic features of healthy and fully repaired tendon tissue. To investigate these three aspects, first we quantified the frequency distribution of nuclei with different flatness factor. This was performed by estimating the nuclear aspect ratio (NAR) as NAR, lower than 0.1 corresponds to completely flattened/elongated nucleus of differentiated tenocyte-like cell. We found a tendency that the frequency of NAR lower than 0.1 in hMSC-Scx group was above 20% and thus higher than that of ED and hMSC-Mock groups ([Figure 2B](#)). Next, we studied and displayed the frequency of the nuclear deviations of approximately 200 nuclei/group in order to assess the cellular alignments within the Achilles tendons of the four study groups ([Figure 2C](#)). In a NT more than 70% of the nuclei are aligned in the range of 0–10° angles to the longitudinal axis, another 25% of the nuclei are in the range of 10–20° angles. Interestingly, only in the hMSC-Scx group about 80% of the nuclei were within the angular deviation range of 0–20°, while in the ED and hMSC-Mock groups more than 50% of the nuclei had angular deviation of 30–70°. This finding became even more apparent when we calculated the average and maxi-

imum values of the nuclear orientation angles, which are shown in the circular sectors (Figure 2C). Only the hMSC-Scx group had average and maximum values very close to that of the control NT group. Last, we analyzed by automated nuclear counting the tendon cell density since at the time period of 16 weeks post-surgery low cell density indicates advanced remodeling phase and overall healing process (Figure 2D). The cell densities within the engineered area of ED, hMSC-Mock and hMSC-Scx groups were higher than the NT group, still the cellular density of hMSC-Scx Achilles tendons was significantly lower than the other two injured groups (Figure 2D).

Scleraxis-programmed tendon progenitors reduce the ectopic bone formation

Our study in 2013 [18] revealed in-tendinous calcification in the repaired Achilles tendons, which we discussed is due to altered biomechanics of the injured tissue. Hence, it was very critical in our new model to examine if the implantation of hMSC-Scx cell will result in lowering the ectopic calcification. Using x-ray imaging and subsequent quantification, we can clearly demonstrate that indeed the hMSC-Scx group had the least degree of calcification in comparison to the ED and hMSC-Mock groups (Figure 3A & B). To further verify this substantial result we also performed immunostaining for the classical bone-marker osteopontin, which confirmed that the hMSC-Scx group had very negligible and scars osteopontin-positive areas (Figure 3C).

Since bone formation is closely related to vascularization, we investigated for endothelial and macrophage-rich areas by immunohistochemistry against CD31 and CD68, respectively (Figure 3D & E). In the ED and hMSC-Mock groups, vessels were observed in the large calcified areas and in the tendon periphery, while in the NT and hMSC-Scx groups vessels were rare and only at the tendon periphery and sheets. CD68-expressing cells were very seldomly observed within vessels in the ED, hMSC-Mock and hMSC-Scx groups without any detectable difference, suggesting their involvement in the gradual vessel obliteration rather than in inflammation.

Scleraxis-programmed tendon progenitors aid in enhanced tendon tissue maturation

Increase of collagen I to collagen III ratio, development of very organized collagen fibers with strong birefringence and enrichment of aligned and denser elastin fibers are hallmarks of advanced remodeling and late maturation phase of the tendon repair process [7]. This is paralleled with interchange of certain proteoglycans in the tendon matrix, namely reduction in biglycan

(regulator of early and thin collagen fibril formation) and upregulation of decorin and lumican levels (regulators of late and thick collagen fibril formation) as well as a general drop in the deposition of mucopolysaccharides and nontendinous glycosaminoglycans/proteoglycans [25,26]. Hence, to study whether implantation of hMSC-Scx cells leads to faster tendon healing in terms of quicker entry into the maturation repair phase of the remodeling stage, we first analyzed the overall content of mucopolysaccharides and cartilage-related glycosaminoglycans in the Achilles tendons of all groups by PAS and S.O./F.G. staining, respectively (Figure 4A & B). Among the injured groups, the ED group showed very strong positive PAS staining as well as several S.O/F.G-rich areas suggesting that the grafting of cells gave an advantage in the tendon healing of the corresponding study groups. This inclination was further confirmed by polarized light microscopy analyses which demonstrated increased collagen birefringence areas, equivalent of more mature collagen fibers, in the Achilles tendons from the hMSC-Mock and hMSC-Scx groups compared with the ED (Figure 5A & B). Last, detailed immunohistochemistry studies of six different antigens, precisely collagen type I and III, biglycan, decorin, lumican and elastin, revealed that their expression pattern in hMSC-Scx group resembled that of the NT group (Figure 6). This group had higher collagen I, decorin, lumican and elastin levels than the ED and hMSC-Mock groups. Very apparent difference was observed with regards to biglycan levels: ED group exhibited very high expression signals, while implantation of either hMSC-Mock or hMSC-Scx cells resulted in biglycan expression comparable to the levels in the NT group.

Altogether, based on our immunohistochemistry data, and together with the above-described results, we firmly concluded that implantation scaffold-free delivery of hMSC-Scx cells leads to advanced and superior healing in a very clinically relevant *in vivo* model of full-size Achilles tendon injury.

The implanted human cells integrate & can survive 16 weeks *in vivo*

To examine whether the implanted hMSC-Mock and hMSC-Scx cells were integrated in the Achilles tendons and a portion of them has survived during the given healing period of 16 weeks, we performed first human-specific PCR on genomic DNA isolated from cryosections of the three injured groups, wherein the ED group served as negative control. In Figure 7A it is shown that in 9 out of 10 animals we detected human DNA only in the study groups that were implanted with cells. Next, by performing immunohistochemistry against the human HLA-ABC epitope, we localized

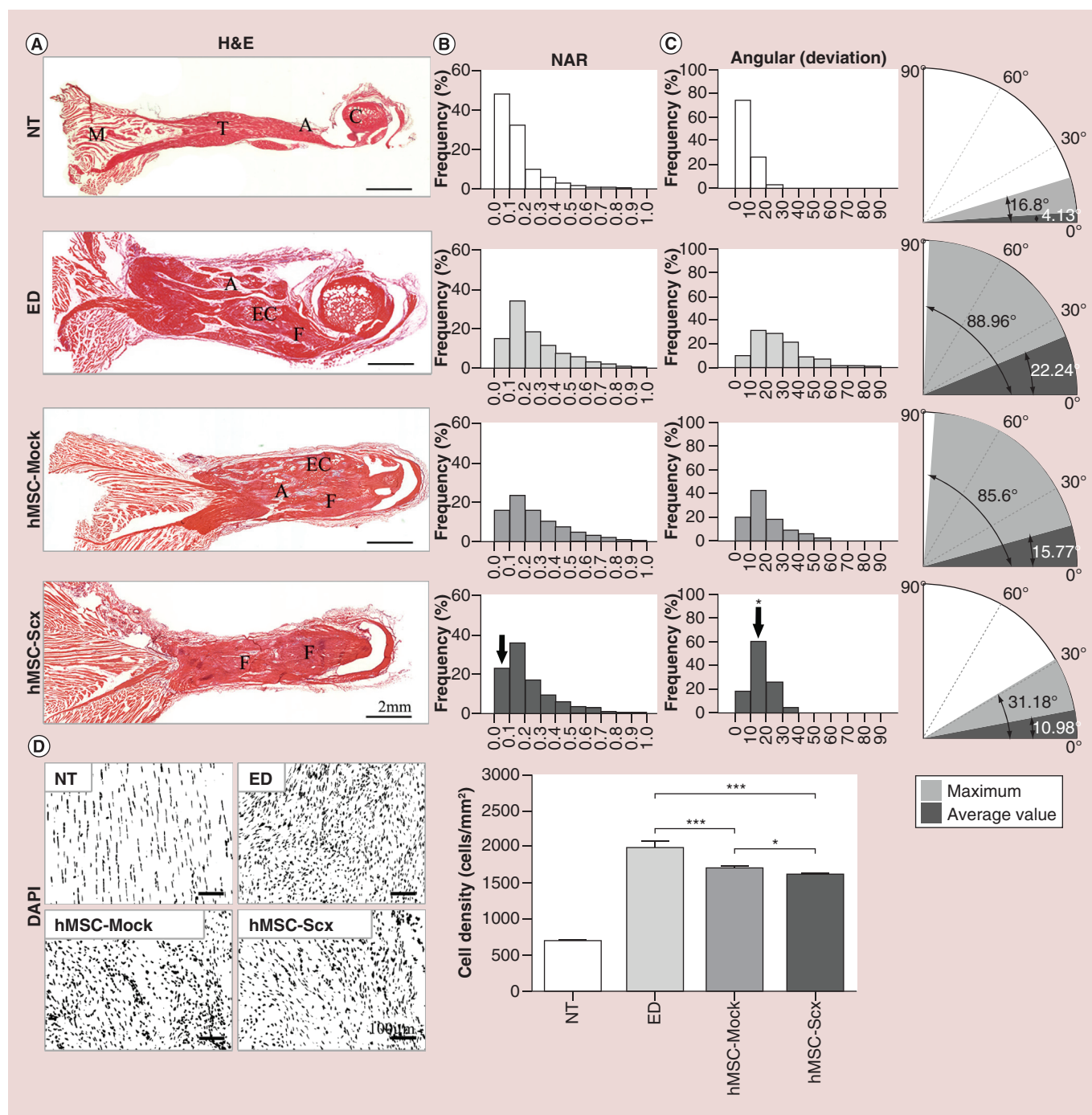


Figure 2. Detailed analyses of tendon tissue organization, nuclear aspect ratio, angular deviation and density. (A) Representative H&E mosaic images for each study group displayed different substructures in the Achilles tendons, the extent of which were scored in Table 1. (B) Frequency distribution of nuclear aspect ratio (NAR) (width vs length of nucleus). The black arrow indicates a tendency in NAR 0.0–0.1 increase in hMSC-Scx versus ED. (C) Frequency distribution and circular sector of maximum and average value of angular deviation (angle between individual nuclear axis and tendon tissue longitudinal axis). The black arrow indicates * $p = 0.05$ of hMSC-Scx versus ED in 10°–20° deviation. (D) Representative black and white-converted images of DAPI staining and chart of calculated cell density. Bar charts present mean \pm standard deviation; *** $p = 0.0001$ hMSC-Scx versus ED, *** $p = 0.0006$ hMSC-Mock versus ED and * $p = 0.05$ of hMSC-Scx versus Mock. Six to nine randomly taken 20 \times magnification images per section of three to five animals per group were analyzed and a total number of approximately 2000 nuclei were measured for NAR and density, while approximately 200 nuclei per group were evaluated for angular deviation.

A: Adipose areas; C: Calcaneus bone; EC: Ectopic calcification; F: Poor fibrous tissue; M: Muscle; T: Organized collagen fibers.

single human cells incorporated within the proper of the Achilles tendons in hMSC-Mock and hMSC-Scx groups (Figure 7B).

Discussion

Due to the global population aging and weak regenerative potential of tendons, age-related degeneration, various tenopathies, traumatic and sport ruptures, tendon conditions still present major challenges to orthopedic

medicine. A novel approach to treat tendon injuries is with tissue engineering, in which cells, alone or in combination with a scaffold, are introduced into the defect in order to enhance the repair and restore the original tissue function faster. In this approach a critical step is to identify the most appropriate cell type.

At present, the preferable cell types for musculoskeletal tissue engineering are adult MSC derived from bone marrow (BM) or adipose tissue (AD) [27–29], as in

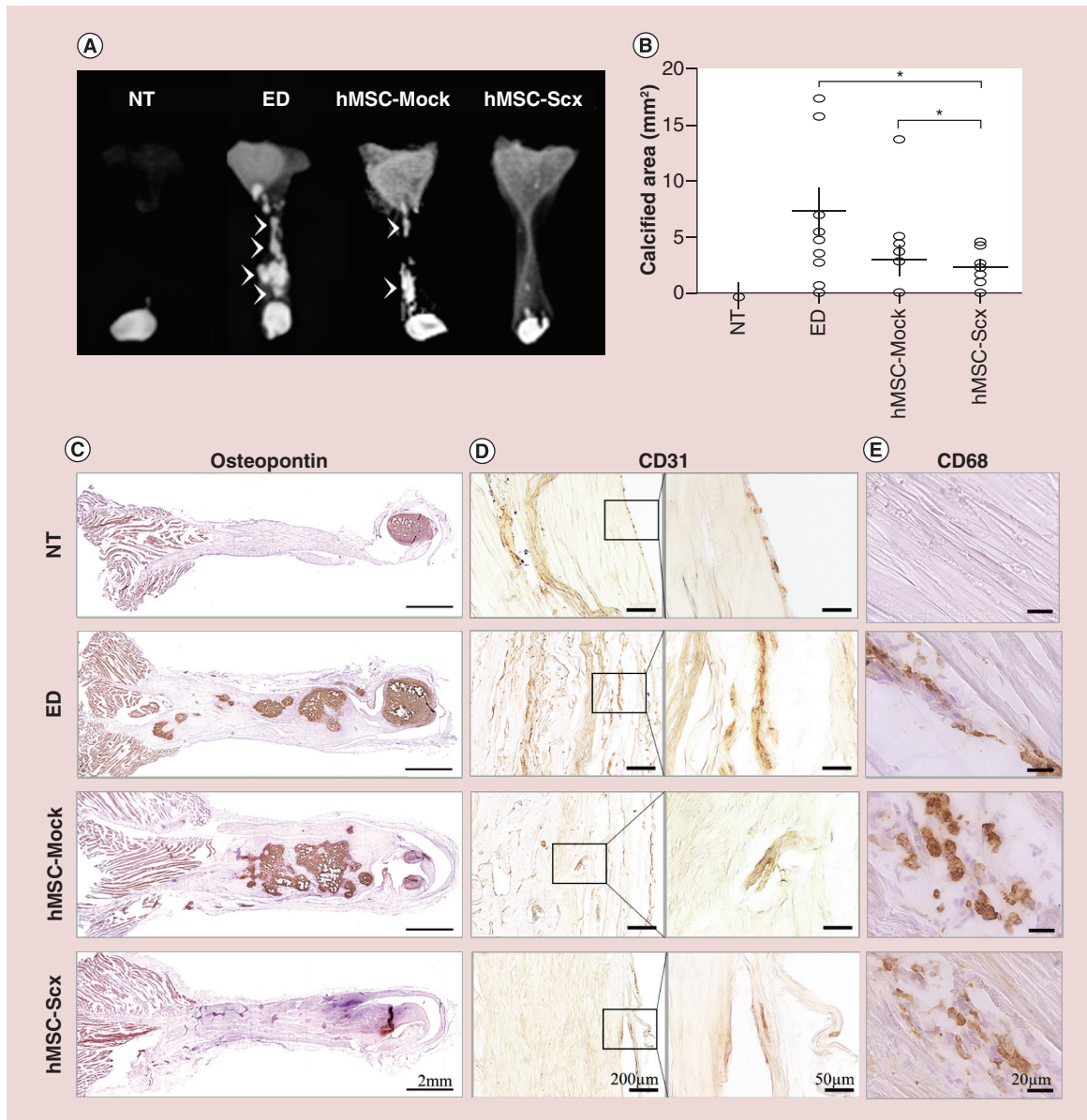


Figure 3. Evaluation ectopic calcification and vasculature within the Achilles tendons of the four study groups. (A) Representative x-ray images (calcification indicated with arrow heads) and (B) plot of total calcified area in each Achilles tendon measured from the x-ray images. Mean values \pm standard deviations (10 animals/group) are included as lines in the plot; * $p = 0.05$ hMSC-Scx versus ED and hMSC-Scx versus Mock. Representative images of the bone-osteopontin (C), the vessel/endothelial CD31 (left panels are 10x magnification, right panels are 40x magnification) (D) and the macrophage CD68 markers (E). Positively stained areas are in brownish color. Stainings were reproduced at least thrice independently and for each antigen sections from three to five animals/group were analyzed.

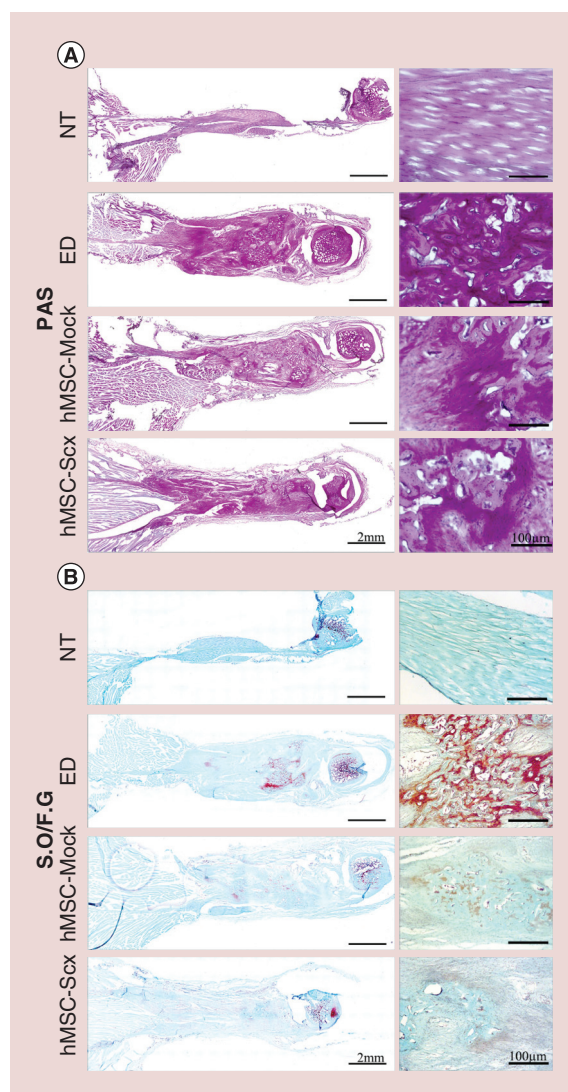


Figure 4. Analyses of mucopolysaccharides depositions and cartilage glycosaminoglycans-rich areas in the Achilles tendons of the four study groups.

Representative mosaic and magnified images of (A) Periodic Acid-Schiff (PAS, dark purple color) and (B) Safranin-O/Fast Green (S.O, orange to red color/F.G, green to light blue color) stainings. Both staining times were reproduced at least thrice independently with sections from five animals/group.

particular the BM-MSC, being the best characterized, are still the most common cell source, because they are easily accessible and expandable, and can be differentiated applying standardized protocols to at least three different mesenchymal lineages. However, there are several difficulties limiting the use of BM-MSC in tendon repair, such as ectopic ossification, bigger scar formation linked to high risk of adhesions and reduced MSC quality with donor age [30]. The results of our model demonstrated, in comparison to nonimplanted injured tendons, that delivery of MSC reduces the rate of in-tendinous ossification; however, among the two

different cell types the hMSC-Scx group was superior. AD-MSC have become an alternative, very attractive cell source derived from plastic surgery, because of their low morbidity isolation procedure as well as lower risk of ectopic bone formation [30]. Studies have concluded a better collagen fiber organization and overall improvement of the tendon structure when using AD-MSC for tendon therapy [31,32]; however, this cell type also faces few challenges including endogenous tendency toward adipocyte differentiation, purity levels of the primary cells and the possible side effects on the AD-MSC quality regarding background metabolic conditions [33]. For the above reasons, in the recent years, the field has turned its attention to tendon-resident stem/progenitor cells (TSPC) [13,34], which have been suggested to play a critical role in the proliferative and remodeling phase of tendon healing [11] and have the native propensity to terminally differentiate in tendon-specific cells [11]. Indeed in our previous study, using the same full size Achilles tendon injury model, the rat tendon-derived population outperformed the classical BM-derived cells in histology and biomechanical analyzes [18]. Several hurdles are associated with the implementation of TSPC in tendon repair. On the technical/methodological side their purification and validation has proven difficult due to the lack of TSPC markers and their extended expansion has been linked to considerable phenotypic drift *in vitro* [35]. On the clinical side, the availability of tendon biopsies for TSPC isolation remains almost an unresolvable issue that is directly concomitant with patient comorbidity and longer preparatory procedures prior treatment, an issue that is further amplified with possible TSPC complications due to patient aging and/or various disease conditions [13]. To overcome the difficulties associated with the use of TSPC, in 2012 we reported an alternative way to generate tendon progenitors based on the one-step programming of the classical BM-derived MSC via the ectopic expression of the leading tendon transcription factor *Scx* [17]. It should be noted that our single cell-derived lines are a very useful standardized experimental model. For true clinical applications, it should be explored if transient *Scx* delivery in autologous MSC is sufficient for their stable programming into the tendon lineage. Others have shown alternative ways to induce tenogenic commitment of hMSC; for example, overexpression of the late tendon marker *EGR1* (early growth response-1) or *BMP2* (bone morphogenetic protein 2)/*Smad8* or combination of *BMP12* and *BMP13* [36–38]. Our previous work showed that subsequent to the involvement of the single early tendon factor *Scx*, hMSC underwent several major changes, comprising of reduced self-renewal, restricted multipotential and upregulated expression

of tendon-related genes, which collectively advocated their successful conversion into tendon progenitors [17].

Here, we can report for the first time that after *in vivo* implantation, our hMSC-Scx cells not only can form tendon-like structures, but can directly contribute to enhanced tendon healing in a clinically relevant Achilles tendon injury model. Based on two different techniques for *in vivo* tracking, namely forensic genomic PCR and antibody-based detection, we could demonstrate that the human cells are retained within the implanted area and that a portion of these cells or their progeny can survive as long as 16 weeks post-surgery. Moreover, via a combination of tissue morphometric, x-ray, polarized light microscopic and various histological and immunohistological analyses of proteins critical in tendon repair process we clearly showed that the hMSC-Scx group had smaller tendon diameter, negligible ectopic calcification and advanced cellular organization and matrix maturation of the injured tendons, making this study group the closest to the native noninjured tendon. Hence, we concluded that hMSC-Scx implantation aids in advanced and superior remodeling stage of the tendon healing. Based on our encouraging results, we will aim in follow-up studies to additionally optimize the application of hMSC-Scx cells in terms of: developing a suitable tendon-specific scaffold for cell delivery in order to further augment the repair outcome; evaluating their potential in a big animal model followed by end point biomechanical testing in order to approximate to the tendon dimensions of patient clinical scenarios; and establishing a transient viral or nonviral approach for *Scx*-programming to surpass the safety concerns related to genetic modification of donor-derived human cells.

The positive effect of *Scx* in tendon healing has been suggested by three other studies. Gulotta *et al.* applied a transient adenoviral expression of *Scx* in MSC to treat tendon-bone insertions of detached by surgical cut rat rotator cuffs, and evaluated tendon healing in the proliferative phase of 2 and 4 weeks. Despite the limited histological evaluation and lack of cell characterization and tracking, the main result was improved biomechanical strength in the *Scx* group [19]. Next, Tan *et al.* utilized, similar to our study, lentiviral delivery of *Scx*, but together with green fluorescent protein (GFP), in rat tendon-derived cells, in combination with fibrin glue and compared their healing potential to Mock (with GFP) and nontransduced cells in a patellar tendon window injury model with terminal analyses at 2, 4 and 8 weeks. The authors concluded that the doubly transgenic *Scx*/GFP tendon cells in comparison to Mock/GFP cells promoted tendon healing at the early stage. However, the data also suggested that the extent of repair after *Scx*/GFP cell implantation is much com-

parable to that of the nontransduced cells. Hence, it remains questionable if overexpression of *Scx* in native tendon-derived cells is beneficial and, as discussed above, a difficulty in the implication of such cells is the associated complications with their obtainment from patients' prior therapy. In addition, it should be considered that in window defect models the neighboring tendon regions stay intact, thus it is difficult to segregate the impact of local stem/progenitor cells to that of the implanted cells [21]. Last, Chen *et al.* tested the use of human embryonic stem cells (hESC), which were first differentiated to MSC, then engineered via lentivirus to express *Scx* and loaded onto silk-collagen sponge for repair of a gap wound model in rat Achilles tendons at 2 and 4 weeks [20]. The *in vitro* pheno-

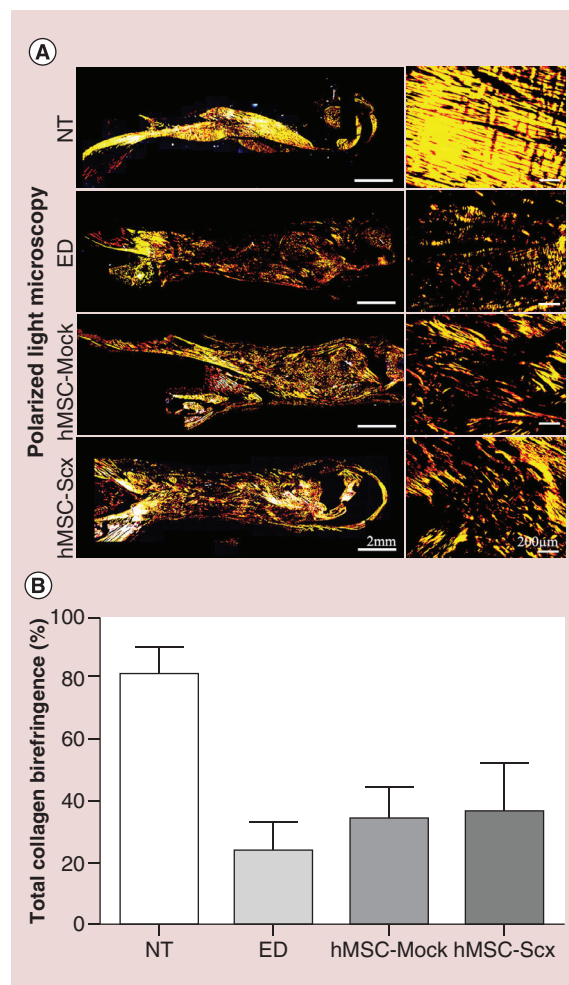


Figure 5. Microscopic evaluation and quantification of mature collagen fiber areas in the Achilles tendons. (A) Representative mosaic and magnified images of H&E-stained Achilles tendons under polarized light, wherein highly organized collagen fibers are visible in orange to yellow colors. (B) Quantification of total collagen birefringence (in percentage of total tendon area). Chart shows means \pm standard deviations of four animals/group.

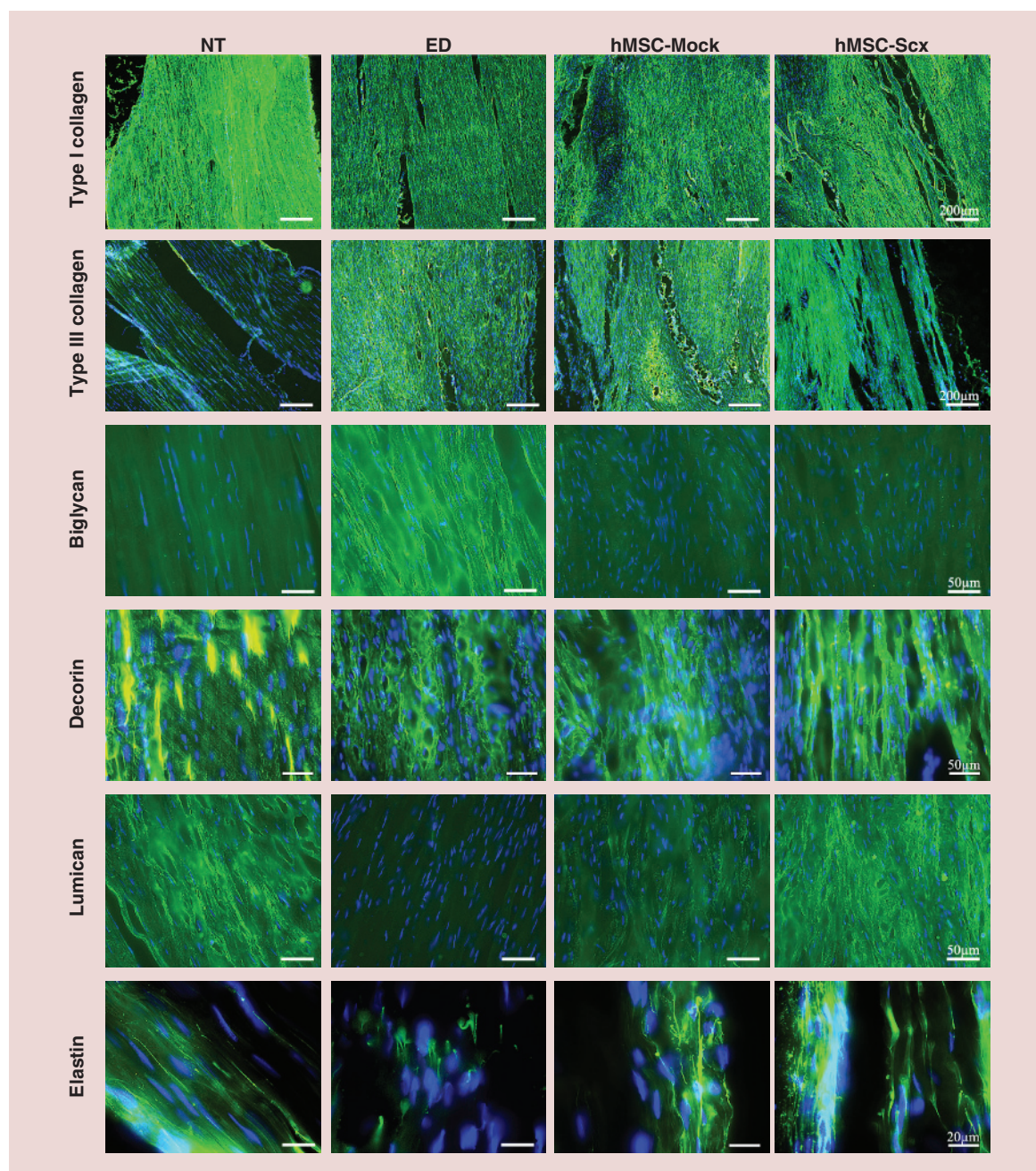


Figure 6. Immunostainings of the tendon-related matrix proteins: collagen type I and III, biglycan, decorin, lumican and elastin (all in green color) and nuclear counterstain DAPI (in blue). All stainings were reproduced at least thrice independently including sections from three to five animals/group.

typization of the hESC-MSC-Scx cells is in line with our first study [17]. At the two evaluated time points of early tendon healing, the Scx group was superior in terms of tissue organization and elastic modulus to the control GFP-transduced hESC-MSC group; it is unclear how approximating are the engineered tendons to noninjured since such were not included for direct comparison. Furthermore, since tendon repair is a time consuming process, the evaluation of the long-

term effects of the cell therapy on tendon composition and strength, functional gain and repeated performance or possible injury reoccurrence has to become a key goal in future studies. In sum, our investigation provides novel and promising findings that a scaffold-free delivery of hMSC-Scx cells for the treatment of a clinically relevant Achilles tendon injury model results in advanced late remodeling stage and overall enhancement of the tendon repair. Hence, we and others can

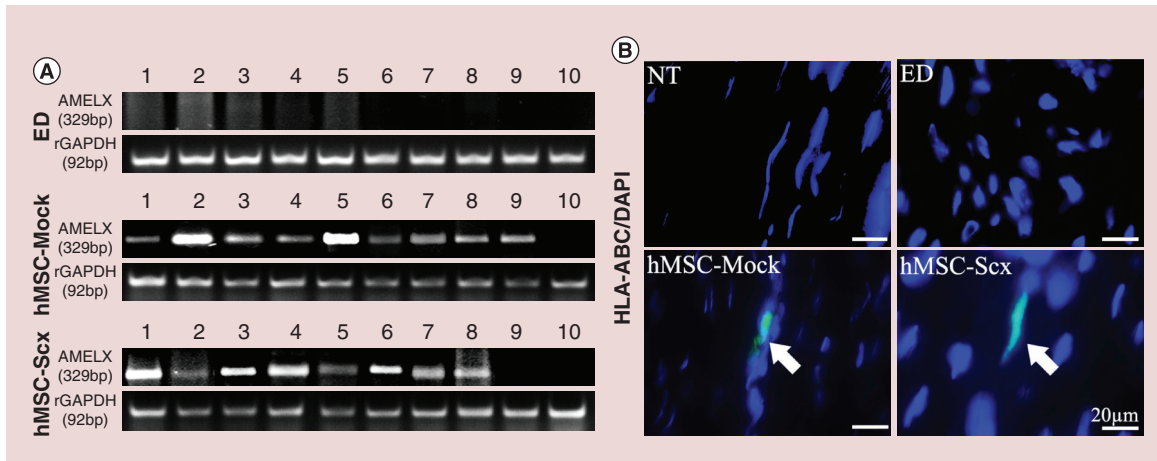


Figure 7. Detection of the implanted human cells in the rat Achilles tendons after 16 weeks *in vivo*. (A) By PCR for the human-specific amelogenin X (AMELX) gene using genomic DNA isolated from tendon tissue sections. Size of PCR product: 329 bp, and for simplicity not the actual animal number, but numbers 1–10 are given in the figure. For loading control PCR for rGAPDH was carried out. Size of PCR product: 92 bp. (B) By immunostaining for the human-specific antigen (HLA)-ABC. In the representative images positive human cells are in green color and the nuclear counterstain DAPI in blue color. PCR and stainings were reproduced twice with samples from 10 and five animals, respectively.

strongly propose *Scx*-delivery as a therapeutic strategy to augment tendon healing.

Conclusion & future perspective

Scaffold-free hMSC-*Scx* cells implantation in injured Achilles tendons resulted in smaller size, negligible intertendinous ossification and advanced tissue maturation of the newly formed tendon, which collectively suggest that hMSC-*Scx* cells exert strong tendon-inducing and differentiating properties making them very well suited for reconstruction of full-size tendon defects. In future, in order for hMSC-*Scx* cells, or variants of this technology, to become a factual clinical tool in orthopedics several open questions have to be addressed,

such as possible combination and optimization with biomaterials/carriers; understanding the exact fate and survival rate of implanted cells; examining stable programming via transient *Scx* expression in autologous cells; long-term functional and biomechanical performance tests in big animal models without immunosuppression; safety inspections behind the cell manipulation; and precise estimation of the time frame from patient hospitalization, preparation of the donors cells to exact point and technique of cell implantation. We believe that the present and forthcoming results on the application of *Scx*-based cell therapy can lead to improve the outcome of tendon surgeries.

Executive summary

- We explored the *in vivo* repair potential of tendon progenitors generated by a direct programming of mesenchymal stem cells with the transcription factor Scleraxis (hMSC-*Scx* cells).
- We utilized our clinically relevant Achilles tendon injury model and implanted scaffold-free hMSC-*Scx* or control hMSC-Mock cells in the injured tendons; the tendon healing was compared with empty defect (ED) and noninjured, native tendons (NT) at 16 weeks post-surgery, a time point corresponding to the late remodeling phase of the repair process.
- Via combination of morphometric, x-ray, polarized light microscopic and various histological and immunohistological analyses we found that the repaired tendons of hMSC-*Scx* group had smaller tendon diameters, negligible ectopic calcification and advanced cellular organization and matrix maturation, hence making this study group the closest to the NT.
- Using two different methods for *in vivo* tracking, we also demonstrated that the human cells are retained within the implanted area and that a portion of these cells, or their progenitors, can survive 16 weeks *in vivo*, suggesting their direct contribution to the healing.
- In sum, we can report that scaffold-free hMSC-*Scx* cell implantation aids in advanced and superior remodeling stage of the tendon healing and can be foreseen, after additional optimization steps, as a novel approach for therapy of tendon injuries.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: <http://www.futuremedicine.com/doi/full/10.2217/NNM.16.34>

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations (approved animal grant no. 55.2-1-54-2531-58-08 by the Animal Care and Use Committee of the Bavarian Government).

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5. Publication 2

Periodontal ligament cells as alternative source for cell-based therapy of tendon injuries: in vivo study of full-size Achilles tendon defect in a rat model

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PERIODONTAL LIGAMENT CELLS AS ALTERNATIVE SOURCE FOR CELL-BASED THERAPY OF TENDON INJURIES: *IN VIVO* STUDY OF FULL-SIZE ACHILLES TENDON DEFECT IN A RAT MODEL

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Abstract

Tendon's natural healing potential is extremely low and inefficient, with significant dysfunction and disability due to hypocellularity and hypovascularity of tendon tissues. The application of stem cells can aid in significantly enhanced repair of tendon rupture; therefore, the main aim of this study is to assess the potential of using periodontal ligament cells (PDL), usually obtained from patients undergoing orthodontic treatment, as a novel cell source for cell-based therapy for tendon injuries in a clinically relevant rat full-size Achilles tendon defect. In addition, the study compares the differences between the healing effects of Achilles tendon-derived cells (AT) *versus* PDL and, hence, comprises of four experimental groups, native tendon (NT), empty defect (ED), PDL and human AT (hAT). The tendon healing in each group was assessed in the late remodelling phase at 16 weeks after surgery using a combination of methods, including evaluation of gross morphological appearance; various histological and immunohistological stainings; and detailed analyses of cell morphometry. Based on these outcome measures, PDL cell-implanted tendons exhibited not only advanced tissue maturation, less ectopic fibrocartilage formation, more organised collagen fibres, tendon matrix expression corresponding to the final healing stage, and better cell-morphometry parameters when compared with the ED group, but were also very similar to the tendons treated with hAT-derived cells. Taken together, our study clearly demonstrates the feasibility of using PDL cells as a novel cell source for tendon repair and strongly recommends this cell type for the future development of innovative regenerative applications for treatment of different tendon or ligament pathologies.

Keywords: Periodontal ligament, Achilles tendon, cell-based therapy, tendon repair, *in vivo*, immunohistochemistry, cell morphometry.

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Introduction

Tendons possess the capability to heal injuries in a process controlled primarily by local tendon cells, invading blood-derived cells and inducing expression changes in the surrounding extracellular matrix. Immediately after tendon injury, a scar formation is initiated, containing three overlapping stages: (1) tissue inflammation, (2) cell proliferation and (3) tissue remodelling (Docheva *et al.*, 2015). The final remodelling stage is time consuming and is characterised by a decrease in cellularity/density, significant cell shape flattening and almost perfect cell alignment to the axis of mechanical stretch, which is accompanied by maturation of collagen type I and elastic fibrils. Additional features of this stage are a decrease in biglycan and an increase in decorin and lumican protein expression (Juneja and Veillette, 2013). In parallel, collagen and elastic fibres start to organise along the longitudinal axis of the tendon, thereby restoring tendon stiffness and tensile strength (Sharma and Maffulli, 2005a).

At present the options to heal injured tendons mainly consist of surgical intervention, tendon/ligament transfer or implantation of synthetic materials; however, they frequently result in unsatisfactory outcomes (Simonson *et al.*, 2016). Hence, repair of tendon defects due to trauma or tendon degeneration remains as a great challenge to orthopaedic medicine. A promising new development of regenerative medicine is cell-based therapy, in which mostly stem cells are directly implanted or used for manufacturing of organoids – mimicking the original state of injured/diseased tissue. Such approach has been experimentally explored also for therapy of tendon injuries (Docheva *et al.*, 2015). Scientists have mainly focused on two favourable cell types, namely bone marrow mesenchymal stem cells (BM-MSCs) and adipose-derived mesenchymal stem cells (AD-MSCs), because they are the best characterised and also fairly simple to obtain and expand *in vitro*. Interestingly, the most of the pre-clinical animal studies concluded that MSC delivery can lead to increased cell proliferation, but these cells often differentiated towards osteoblasts or adipocytes within the tendon area, suggesting their inherent preference to commit

in the original lineage of the tissue from which they were isolated (Docheva *et al.*, 2015). Subsequently, the native to the tendon-tenocytes, tendon stem/progenitor cells or tendon-derived fibroblasts became of great interest and their isolation has been successfully carried out (Bi *et al.*, 2007). However, the use of such autologous tendon-derived cells faces several concerns. For example, the patient has to undergo additional surgery to obtain a tendon biopsy for the purification of tendon cells causing comorbidity. Another concern is if the cells are to be isolated from the injured tendon they might be compromised in terms of quality and function (Docheva *et al.*, 2015; Sharma and Maffulli, 2005b). Furthermore, the expansion of the tendon cells to sufficient numbers for implantation will require several weeks, in which the patient will be on hold or has to revisit the hospital numerous times.

In the last years it has been recognised that MSCs exist in a wide range of tissue types, including dental and ligament tissues (Hakki *et al.*, 2015; Lei *et al.*, 2014; Liu *et al.*, 2015). Periodontal ligament (PDL)-derived cells are of particular importance as in comparison to tendon cells, in a way that they are much more easily accessible through the oral cavity and can be simply harvested especially from extracted teeth when patients have orthodontic treatments (Miura *et al.*, 2003; Seo *et al.*, 2004). Recently, several groups, including our, reported that PDL contains endogenous stem/progenitor cells (Bluteau *et al.*, 2008; Docheva *et al.*, 2010; Ivanovski *et al.*, 2006), which express tendon-specific genes such as scleraxis and tenomodulin (Docheva *et al.*, 2010).

The above findings strongly motivated us to investigate if PDL-derived cells can be used for cell-based therapy of tendon injuries. PDL cells hold several advantages to tendon-derived cells in regenerative applications, namely: easier obtaining and banking; avoiding additional surgery with potential risk for pain and infection and decreasing donor comorbidity. For this we designed not only an *in vivo* testing of the potential of PDL cells to form tendon-like structures, but actually directly implanted the cells in a clinically relevant tendon injury model by utilising our established surgically created full-size tendon defects in rat Achilles tendons. In order to estimate the regenerative capacity of the PDL cells we compared the tendons receiving PDL cells to tendons implanted with Achilles tendon (AT)-derived cells as well as to non-implanted empty defect tendons. We assessed, by evaluation of gross morphological appearance, various histological and immunohistological stainings, and detailed cell morphometric analyses, how advanced is the healing process at 16 weeks after surgery and implantation, a time point corresponding to the late tendon repair phase. The results presented below strongly demonstrated that PDL cells contributed to tendon repair, which was very comparable to that of AT cells and far more superior to non-implanted tendons, hence concluding their feasibility as alternative cell source holding many advantages for therapy of tendon injuries.

Materials and Methods

Cell preparation prior implantation

In the study two standard cell lines characterised before, namely PDL-(hTERT) cell line from Docheva *et al.* (2010) and human AT-(hTERT) cell line derivative from Kohler *et al.* (2013) were implemented. Both cell lines were cultured in DMEM/Ham's F-12 (1:1 mixture) medium supplemented with stable glutamine (365.3 mg/L), 1×10^{-6} M amino acids (all from Biochrom, Berlin, Germany), 10 % FBS and 1 % L-ascorbic acid-2-phosphate (both from Sigma-Aldrich, Steinheim, Germany) in a humidified incubator at constant 37 °C and 5 % CO₂. Prior to implantation, the cells were expanded in three consecutive passages to 12×10^6 corresponding to 1×10^6 cells per animal.

Animal model and surgical procedure

The surgical procedures were carried out according to our established *in vivo* model (Pietschmann *et al.*, 2013). The procedures including animal handling prior, during and after surgery were approved by the Animal Care and Use Committee of the Bavarian Government (Animal Grant Nr. 55.2-1-54-2531-58-08). Thirty athymic female Foxn1nu rats were purchased from Charles River Laboratories (Sulzfeld, Germany). Four different study groups were randomly selected (with ten animals per group): (1) native tendon (NT), non-injured left Achilles tendon; (2) empty defect (ED) control group, consisting of injured right Achilles tendon without cell implantation; (3) and (4) in which the animals received one-shot implantation of PDL-(PDL group) or hAT-cell pellets (hAT group) within injured right Achilles tendon, respectively. In the experimental design, in order to reduce numbers of animals sacrificed in the study, injury was applied to the right leg, while the left, non-injured was used as native tendon control.

In brief, the surgical procedure consisted of: (1) animal anaesthesia was carried out; (2) the right Achilles tendon was exposed with a straight skin incision; (3) three millimetres of tissue from the Achilles tendon proper were resected by a surgical removal; (4) the defect was reconnected and stabilised with monofilament PDS (polydioxanone) 5-0 suture material by Kirchmayr-Kessler suture; (5) the original gap of 3 mm was kept and no end-to-end was done in order not to shorten the tendon; (6) a three dimensional cell pellet of 1×10^6 cells was administrated into the defect site with a sterile pipette tip; and (7) the skin was sutured. After surgery the rats were allowed to walk freely in their cages and evaluated for tendon repair after 16 weeks. For this all animals were sacrificed by euthanasia and gastrocnemius muscle-Achilles tendon-calcaneus bone units were retrieved after skin incision, imaged and total length of the total Achilles tendons and width of tendon proper of the Achilles tendons were manually measured.

Histology

Processing

The explanted gastrocnemius muscle-Achilles tendon-calcaneus bone units were fixed either in 95 % ethanol/ 5 % absolute acetic acid overnight at 4 °C, or in 4 %

paraformaldehyde (PFA) (Merck, Darmstadt, Germany) (five animals in each fixation). After fixation, specimens were decalcified in 10 % EDTA/PBS pH 8.0 (Sigma-Aldrich, Munich, Germany) for four weeks at room temperature, then embedded in tissue freezing medium (Jung, Leica, Nussloch, Germany) and cryosectioned at 12 μ m (Microm HM500 OM, Fisher, Walldorf, Germany). Prior use sections were stored at -20°C .

Section hydration prior staining, dehydration and mounting after staining

All the sections before staining were equilibrated to room temperature and hydrated with PBS 3×5 min then changed to deionised water. After following staining, Haematoxylin and Eosin (H&E), Safranin O & Fast Green (S.O/F.G.), Periodic Acid-Schiff (PAS) and 3,3 diaminobenzidine (DAB)-based staining, routine dehydration and mounting were carried out as follows: sections were washed through a graded ethanol series for 5 min each from 70 %, 80 %, 90 %, then 100 % ethanol and xylene (Carl Roth, Karlsruhe, Germany) 2×5 min, and last mounted with Entellan mounting medium (Carl Roth, Karlsruhe, Germany).

H&E

Samples were placed in Haematoxylin (ready for use solution) (Carl Roth, Karlsruhe, Germany) in distilled water for 5 min, rinsed under deionised water for 5 min, washed in tap water for 7 min. Next, samples were immersed in Eosin (ready for use solution) (Carl Roth, Karlsruhe, Germany) for 5 min, changed to distilled water for 30 s, dehydrated and mounted.

S.O/F.G. & quantification

Samples were placed in 0.02 % Fast Green (Sigma-Aldrich, Steinheim, Germany) in 95 % ethanol for 12 min, rinsed under 1 % acetic acid in distilled water for 5 min, immersed in 0.5 % Safranin O (Sigma-Aldrich, Steinheim, Germany) in water for 15 min. Last, samples were rinsed quickly in 90 % and 100 % ethanol and mounted. In order to cover the entire tendon territory, approx. 50 consecutive images/section were taken and digitally stitched with Adobe Photoshop CS5 software (Adobe System, San Jose, CA, USA). Next, total Achilles tendon area (denominator) was measured in pixel with “magic wand” tool and by using “select colour range” tool the red-to-orange-positive pixels (numerator), corresponding to sulphate glycosaminoglycans (GAGs) areas, were automatically quantified. Final data were expressed as a percentage of GAGs and for each study group mean values and standard deviations were calculated.

PAS

The PAS staining kit was obtained from Sigma-Aldrich. Samples were placed in periodic acid solution for 5 min, rinsed 3 times under distilled water, immersed in Schiff's reagent (St. Louis, MO, USA) for 15 min, washed in running tap water for 5 min. Next, samples were stained in haematoxylin solution for 90 s, rinsed in running tap water, dehydrated and mounted.

All stainings were reproduced thrice independently as three to five animals/group were analysed.

Immunohistochemistry

Antibodies

Primary: biglycan (ab58562, 1:30 dilution), collagen I (ab34710, 1:100), collagen III (ab7778, 1:50) and elastin (ab21610, 1:30, all from Abcam, Cambridge, UK); decorin (sc-22753, 1:30) and lumican (sc-33785, 1:50) (both from Santa Cruz, Heidelberg, Germany); osteopontin (MPIIB101, 1:20, DSHB, Iowa City, IA, USA); (HLA)-ABC (M0736, 1:30, Dako, Glostrup, Denmark). Secondary: anti-goat Cy3 (705-165-147, 1:500), anti-rabbit FITC (111-095-144, 1:500), anti-mouse Cy3 (115-165-146, 1:500) (all from Jacksonimmuno, West Grove, PA, USA) and anti-mouse vectastain ABC kit (PK-4002, 1:250, VectorLab, Peterborough, UK).

Immunolabelling procedure

Selected sections were treated with 2 mg/mL hyaluronidase (Sigma-Aldrich, Steinheim, Germany) for 30 min at 37°C . After washing and blocking, either with 2 % skimmed milk (Sigma-Aldrich, Steinheim, Germany) / PBS or 2 % BSA (Sigma-Aldrich, Munich, Germany) / PBS, primary antibodies were applied overnight at 4°C . Next day, corresponding secondary antibodies were used for 1 h. For immunofluorescence-based experiments, sections were shortly counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Life technology, Carlsbad, CA, USA) and mounted with Fluoroshield (Sigma-Aldrich, Munich, Germany). For DAB-based experiments, an initial quenching step was performed using 4 % H_2O_2 /methanol for 30 min at room temperature, and afterwards the classical vectastain ABC kit was used according to the manufacturer's instructions (VectorLab, Eching, Germany), then sections were dehydrated and mounted.

All stainings were reproduced thrice independently with three to five animals/group analysed.

ELISA quantification of collagen type I, biglycan and lumican protein expression

PFA-fixed sections mounted on microscope slides (90 sections each animal, 5 animals per group) were used for protein extraction. Briefly, only Achilles tendon area was collected manually by scalpel. The tissue material was lysed in extraction buffer, composed of 8 M urea (Merck, Hohenbrunn, Germany), 50 mM Tris-HCl (Merck, Darmstadt, Germany), 1 mM EDTA (Carl Roth, Karlsruhe, Germany), 1 mM DTT (BIO-RAD, Munich, Germany), by incubation at 4°C for 30 min, followed by centrifuged for 20 min at $1 \times 10^4 \times g$, and supernatants containing protein extracts were stored at -20°C until further use. Protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo, Rockford, IL, USA) according to the manufacturer's protocol. The protein samples were normalised between animals and further diluted 1:50 in order to lower the antigen concentration to the detection range of the ELISA kits. Commercially available ELISA kits for collagen type I (LS-F4691) biglycan (LS-F15938, both from LifeSpan BioScience, Seattle, WA, USA) and lumican (CSB-EL013234RA, Cusabio Biotech, College Park, MD, USA) were used following the manufacturer's instructions and twice independently reproduced with 5 animals per group.

Quantification of collagen type III

An automated quantitative image analysis was performed as described in McCloy *et al.* (2014) with slight modifications. In brief, using ImageJ (National Institutes of Health, Bethesda, MD, USA) the following algorithm was applied: (1) nine randomly 10× magnification images were taken within the area of injury; (2) area of interest was manually designated using the “drawing/selection” tool; (3) “set measurements” for area, integrated density and mean grey value was selected from the analyse menu; (4) last the corrected total cryosections fluorescence (CTCF) representing the collagen III expression was exported and calculated in Excel (Microsoft) as follows $CTCF = \text{media of integrated density} - \text{media of the selected area} \times \text{mean fluorescence}$. Three animals per group (each with nine images) were analysed.

Elastic fibres scoring

A point-based scoring system for elastic fibres assessment was used according to Hsieh *et al.* (2016) and legend of Table 2. Elastin-stained sections from three animals (each animal represented with 3 to 5 different slides consisting of 3 to 4 sections) were employed for microscopical scoring in a blinded fashion.

Classical microscopy

Photomicrographs of H&E, S.O/F.G., PAS, DAB-based stainings were taken with a colour AxiocamICc3 camera, while images of immunohistochemical stainings were taken with a black/white Axiocam MRm camera, both mounted on Observer Z1 microscope (Carl Zeiss, Jena, Germany).

Polarised microscopy & evaluation

H&E-stained sections from the 4 study groups (3 animals per group and each animal represented with 3 different sections) were imaged using 10× objective supplemented with polarised filter mounted on Axioskope 2 microscope (Carl Zeiss, Jena, Germany). Under this filter, organised collagen type I fibres can be visualised in orange to yellow colours when exposed to polarised light. For final representation, consecutive images of the whole unit of gastrocnemius muscle-Achilles tendon-calcaneus bone were digitally stitched together with Adobe Photoshop CS5 software (Adobe System).

Organised collagen type I fibres have strong birefringence property when exposed to polarised light; hence, they can be visualised in orange to yellow colours using microscopy. Microscopy was performed with 10× objective supplemented with a polarised filter mounted on Axioskope 2 microscope. For optimal imaging the transmission axis of the analyser was with an angle of 54° to the axis of the polariser in the analyses of all samples. In order to cover the entire tendon territory, approx. 50 consecutive images/section were taken manually in a mosaic manner and digitally stitched with Adobe Photoshop CS5 software (Adobe System). Next, total Achilles tendon area (denominator) was measured in pixel with “magic wand” tool and by using “colour selecting” tool the yellow-to-orange-positive pixels (numerator),

corresponding to highly organised collagen areas, were automatically quantified. Final data were expressed as a percentage of collagen birefringence and for each study group mean values and standard deviations were calculated.

Quantification of nuclear aspect ratio and density

Determination of cell density by nuclear count and aspect ratio of nuclei (NAR, defined as the ratio of the minor diameter to the maximal diameter) was performed with the following algorithm: (1) 9 randomly taken 20× magnification DAPI-stained images within the area of injury, 3 mm proximal from the calcaneus, for 3 to 5 animals per group were taken; (2) DAPI images were analysed using ImageJ software v1.49 with installed NII plugin (National Institutes of Health, Bethesda, MD, USA); (3) each image was converted to greyscale by colour threshold adjustment (DAPI-stained areas in black and background in white); (4) the total number of nuclei/image and the minimal and maximal diameter for each nucleus were automatically measured by “analyse particles” tool in NII plugin; (5) the obtained values were exported to an Excel spreadsheet (Microsoft); (6) average cell density per group was expressed by calculating the mean number of nuclei per mm²; (7) NAR was calculated in Excel and distributed by frequency using GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA). Rounded cells are represented by NAR = 1, while flattened cells have NA = 0 and all nuclei/group were assessed calculation of NAR.

Quantification of nuclear orientation

To determine nuclear orientation by estimating angular deviation, H&E-stained images were analysed using the AxioVision V4.8 software (Carl Zeiss, Jena, Germany). Six randomly taken 20× magnification images for 3 to 5 animals per group were analysed and a total number of approx. 200 nuclei/group was evaluated for angular deviation. The angle measurements between the longitudinal axis of the tissue and the major axis of a nucleus were automatically given with the “angular” tool for manually selected nuclei. The angular deviation was distributed by frequency with GraphPad Prism 5 software (GraphPad). Nuclear orientation angle with values of 0° represents a nucleus that is perfectly aligned along the longitudinal axis and as this value increases when the nucleus becomes more angled to the long axis of the tendon.

X-ray analysis

In-tendinous calcification was screened in dissected hind limbs from the four study groups (ten animals/group) using X-ray imaging with a Cabinet X-ray Faxitron (32 kV, 2 mA and 2 s exposure time, Hewlett Packard, McMinnville, OR, USA). The total area of calcification in each animal was digitally quantified from X-ray images using the “polygonal” tool of Image Pro Plus software v4 (Media Cybernetics, San Diego, CA, USA). Mean value and standard deviations were calculated for each study group (ten animals/per group) with GraphPad Prism 5 software (GraphPad).

Data and statistics analysis

Quantitative data and statistical significance analyses were done with GraphPad Prism 5 software (GraphPad). Bar charts show mean values and standard deviations. Multi group statistical testing was performed with one-way ANOVA followed by *post-hoc* Dunnett's test and a *p*-value of < 0.05 was considered statistically significant.

Results

Gross and morphological appearance of Achilles tendons after cell implantation

After 16 weeks of surgery the gross appearance and morphological organisation of the Achilles tendons were assessed by imaging, length and width measurements and H&E stainings. The tendon defect ends were re-connected in all injured groups, including the ED group. However, the Achilles tendons of the PDL and hAT groups had more opalescent tissue appearance in comparison to the ED group (Fig. 1a). Furthermore, according to our quantitative data, these two groups exhibited smaller mean tendon width compared to the ED group, whereas no significant differences were found regarding the mean tendon length (Table 1). H&E-stained mosaic imaging of the four experimental groups were performed (Fig. 1b) and used for examination of the overall morphological organisation and composition of different tissues sub-areas such as tendinous regions (from the NT group), areas of high cellularity, ectopic calcification, and adipose deposition (from the ED group) (Fig. 1c). In the control native Achilles tendons (Fig. 1b, NT), well organised parallel collagen fibres are clearly visible; the tendon tissue is very homogeneous with low number of cells, while some adipose and loose connective tissue can be found on the outer side of the Achilles tendons. In contrast, in the ED group large ossified and vascularised areas surrounded with poorly organised fibrous tissues containing high cell numbers as well as occasionally fatty deposits were characteristic features (Fig. 1b, ED). The cell-implanted PDL (Fig. 1b, PDL) and hAT (Fig. 1b, hAT) groups were very similar to each other and when compared to the NT group, they were clearly larger and more heterogeneous. The Achilles tendons in these groups were mostly composed in the

Table 1. Mean length and width of the four study groups.

Study group	Length [cm]	Width [mm]
NT	1.14 ± 0.12	1.60 ± 0.23
ED	1.32 ± 0.14	3.38 ± 0.59
PDL	1.32 ± 0.10	2.84 ± 0.36*
hAT	1.34 ± 0.14	2.65 ± 0.49**

Values present mean ± standard deviation;

* *p* = 0.03 PDL vs. ED and ** *p* = 0.0074 hAT vs. ED.

periphery of larger tendinous areas with well-organised collagen fibres and in the centre with more fibrous regions with higher cellularity indicating still ongoing tissue remodelling. However, ectopic calcification formation appeared scarce and smaller in size, which represented a major difference between PDL and hAT groups to the ED group. This first assessment of the PDL-implanted Achilles tendons suggested that this group performs comparably to the hAT group, but also is superior to the non-implanted injured tendon group, ED. To fully validate our initial observations, we carried out detailed molecular analyses of the ectopic calcification and tendon matrix composition as well as assessment of characteristic cellular features by cell morphometry quantifications.

Evaluation of ectopic calcification formation

In order to carefully compare the in-tendinous ossification we first analysed the overall content of related to the initial fibrocartilage glycosaminoglycans and mucopolysaccharides by S.O/F.G. and PAS staining of all groups (Fig. 2a and Fig. 2b). A drop in the deposition of non-tendinous glycosaminoglycans/proteoglycans and mucopolysaccharides is an indication for advanced remodelling and entry in the maturation phase of the tendon repair process. Among the three injured groups, S.O/F.G.-rich areas and very strongly positive PAS staining were detected only in the ED group mainly around the ectopic calcification areas. This observation was further confirmed by quantification of S.O-positive area (Table 2). Then, by using an antibody directed against the bone

Table 2. Quantification of S.O.-positive area, calcified area and total collagen birefringence and scoring of elastic fibres.

Study group	S.O. positive area [%]	Calcified area [mm ²]	Total collagen birefringence [%]	Scoring of elastic fibres	
				Content	Morphology
NT	2.83 ± 0.96	0 ± 0	81.34 ± 9.07	4	4
ED	15.46 ± 5.88	3.79 ± 1.04	24.15 ± 9.53	2.25	1.25
PDL	7.50 ± 4.11	2.45 ± 0.73	30.87 ± 8.51	3	3
hAT	11.03 ± 2.59	2.98 ± 0.90	33.88 ± 9.36	3	3.5

Quantification digital image analysis of S.O. stainings, X-ray and polarised light microscopy. Scoring was done in a blinded fashion using the following point system: for content the total amount of elastin fibres (non-mature or mature) compared to NT was estimated by 4 points = 0-25 %, 3 points = 26-50 %, 2 point = 51-75 % and 1 point = 76-100 %, for elastic fibres morphology 4 points = mature, thin parallel elastic fibres, while 1 point = non-mature, thick irregular elastic fibres.

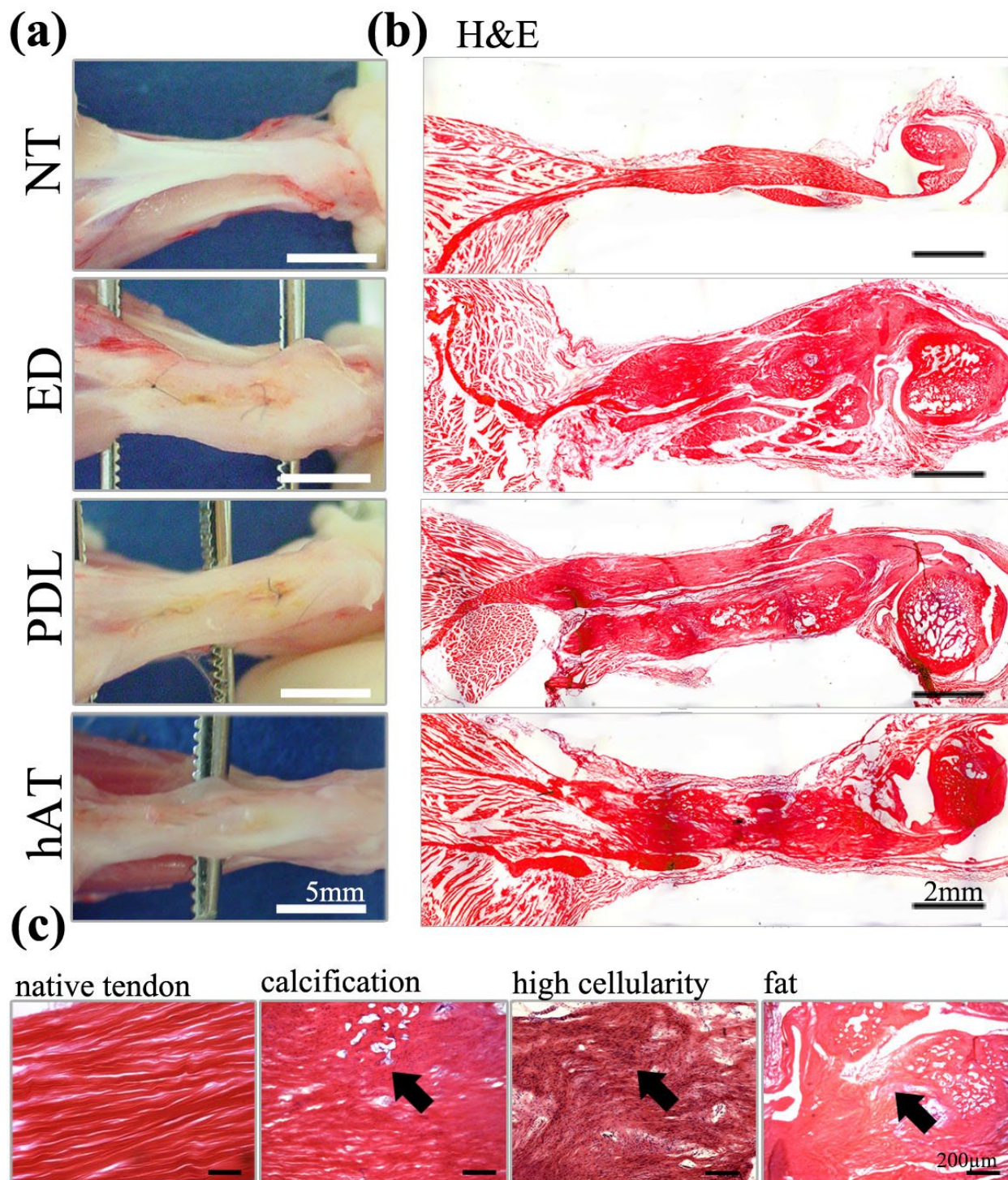


Fig. 1. Overall appearance and tissue morphology of the Achilles tendons in the four study groups. (a) Representative gross morphological images and (b) representative H&E mosaic photomicrographs of NT, ED, and PDL- and hAT-implanted Achilles tendons after 16 weeks of healing. (c) Examples of H&E micrographs demonstrating different tissue sub-areas such as tendinous areas (from NT group), areas of high cellularity, ectopic calcification, and adipose deposition (from ED group) in the Achilles tendon explants.

marker osteopontin (Fig. 2c) and by quantifying the mean area of ectopic calcification on X-ray images of 10 animals per group (Table 2) it was revealed that the dimensions of calcification in PDL and hAT group are very comparable. Due to detected in-group variability, no significant difference but a tendency was found to ED group in terms of ossification. In total, the above analyses revealed that the

implantation of either PDL or hAT cells was advantageous for the tendon healing process.

Expression and organisation of collagen and tendon-related matrix proteins

We analysed the expression of the two major collagens, namely collagen I and III that are critically involved

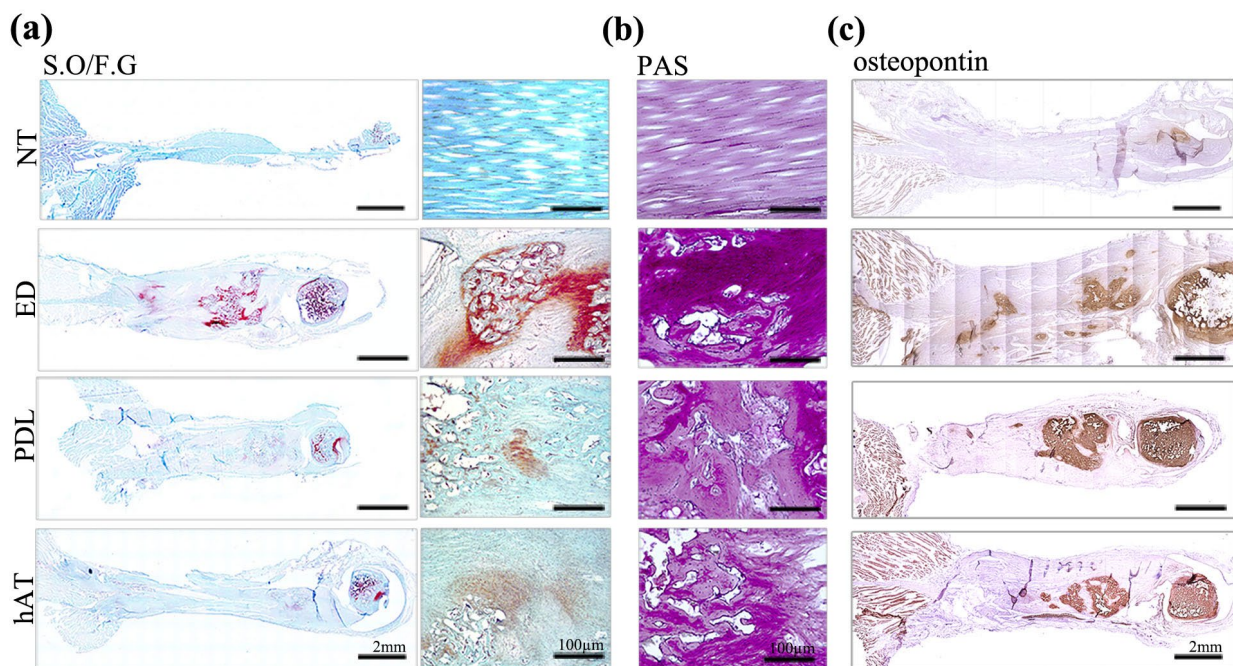


Fig. 2. Analyses of glycosaminoglycans- and mucopolysaccharides-rich areas and ectopic calcification in the Achilles tendons. **(a)** Representative mosaic and magnified images of Safranin O (S.O, orange to red colour)/ Fast Green (F.G., green to light blue colour), **(b)** Periodic Acid-Schiff (PAS, dark purple colour) and **(c)** bone marker protein – osteopontin stainings. All staining were reproduced thrice independently with sections from 5 animals/group.

during tendon healing. Our results showed that the PDL and hAT groups had higher immunofluorescence signals for collagen type I and lower signals for collagen type III than the ED group (Fig. 3a and b). This observation was further confirmed by polarised light microscopy (Fig. 3c) and quantitative analyses (Table 2), demonstrating increased collagen birefringence, equivalent of more superior organised and mature collagen fibres, in the Achilles tendons from the PDL and hAT groups compared to the ED. To quantify collagen type I (Fig. 3d) and III (Fig. 3e) expression, we next carried out ELISA and automated digital image analysis, respectively. The quantitative evaluation demonstrated that the concentration of collagen type I was significantly higher in the hAT group followed by the PDL. With regards to collagen III expression, all injured groups had significantly higher levels than NT group. A tendency for lower collagen III expression in PDL group was detected (Fig. 3e).

Then, immunofluorescence-based studies of four different antigens – biglycan, decorin, lumican and elastin – were carried out (Fig. 4a and Table 2). In general, advanced remodelling and the late maturation phase of the tendon repair process is characterised not only by decreased ratio of collagen type III to collagen type I but also by reduction in biglycan (regulator of early and thin collagen fibril formation) and upregulation of decorin, lumican (regulators of late and thick collagen fibril formation) and elastin levels. Such molecular pattern is typical for the control non-injured NT group. The exact biglycan and lumican protein levels were measured with target-specific ELISA. The obtained quantification data not only confirm the immunofluorescence results, but also revealed a significantly lower biglycan (Fig. 4b) and

significantly higher lumican (Fig. 4c) expression in PDL and hAT groups. Finally, scoring of elastin fibres content and morphology suggested pattern more close to the NT in PDL and hAT groups.

All in all, our analyses of the selected proteins revealed in the PDL group a tissue composition very comparable to hAT group, and furthermore closer to the NT group. In contrast, ED group exhibited high levels of biglycan and low levels of decorin, lumican and elastin. Altogether, based on our investigation outcomes we concluded that the PDL group, similar to the hAT group, has more mature and organised matrix protein composition than the ED group.

Cell morphometric analyses

The above described changes of the tendon matrix in advanced stage of tendon healing are further accompanied with lowering of cell density, cell flattening and parallel alignment to the tensile axis. Therefore, we first evaluated cell density in the four experimental groups by performing nuclear counting on DAPI images and found that the cell densities of PDL- and hAT-implanted Achilles tendons are significantly lower than the ED group (Fig. 5a). Next, we assessed cellular alignment by analysing the frequency distribution of angular degrees of cells within the Achilles tendons of the four study groups. In the NT group more than 95 % of the nuclei were aligned in the range of 0-20° angle to the longitudinal tendon axis. In the PDL and hAT groups about 60 % of the nuclei were within the angular deviation range of 0-20°, while in the ED more than 60 % of the nuclei had angular deviation of above 30° (Fig. 5b). Finally, we quantified the frequency distribution of the nuclear aspect ratio (NAR), which directly reflects the flattening of cells (Fig. 5c), and found in NT group

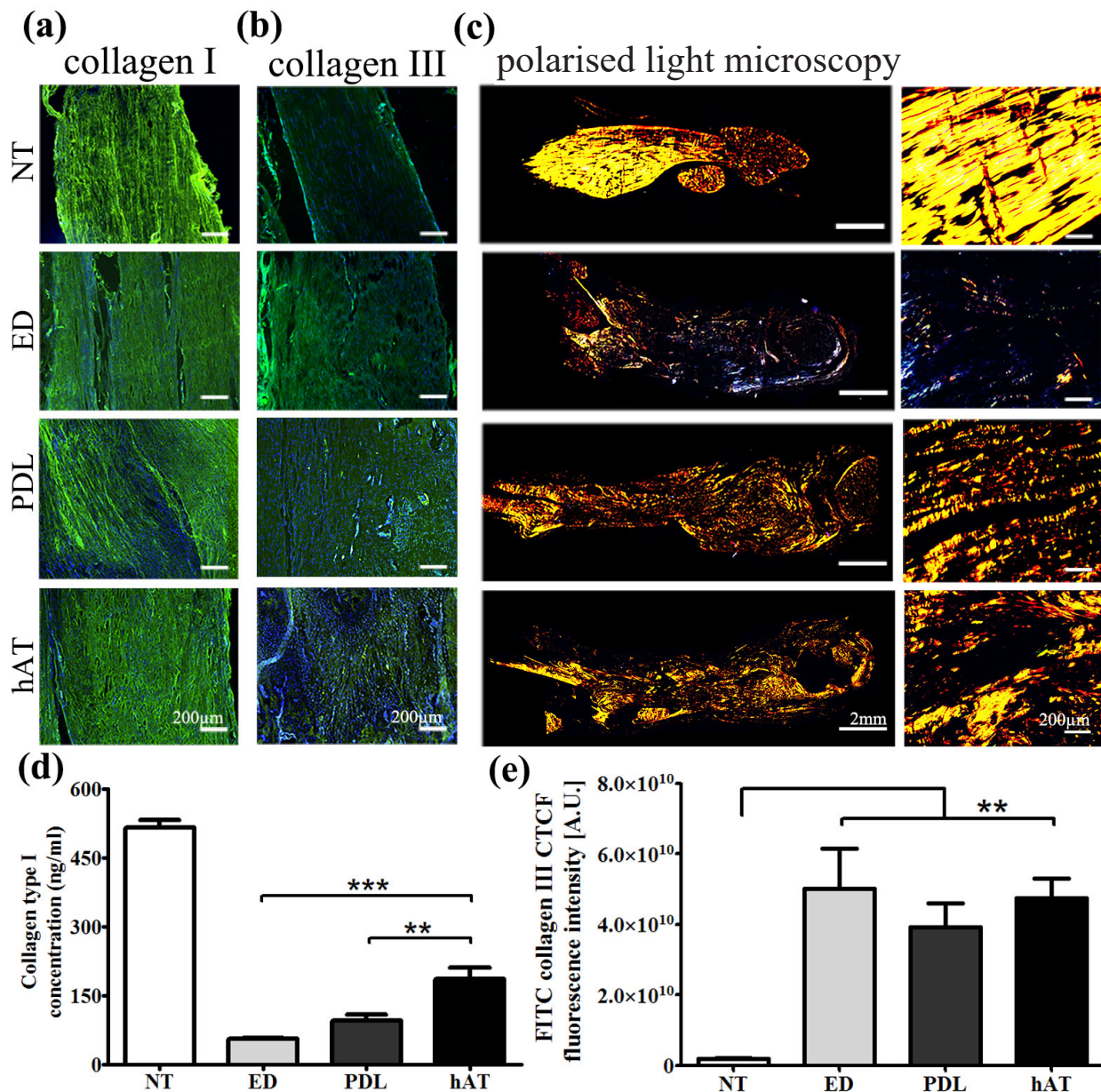


Fig. 3. Expression and organisation of collagen proteins. (a) Collagen type I and (b) Collagen type III immunofluorescent stainings (in green colour) and nuclear counterstain DAPI (in blue). (c) Representative mosaic and magnified images of H&E-stained Achilles tendons under polarised light, wherein highly organised collagen fibres are visible in orange to yellow colours. All stainings were reproduced thrice independently, including sections from 3 to 5 animals/group. (d) Collagen type I levels were assessed by ELISA. Bar charts present mean \pm standard deviation; *** $p < 0.005$, ** $p < 0.01$. (e) The collagen type III protein expression was quantified by automated digital image analyses and expressed as CTCF (corrected total cryosection fluorescence) in arbitrary units (A.U.). Bar charts present mean \pm standard deviation; ** $p = 0.0025$.

that the majority of the cells, approx. 85 %, have NAR of 0.0-0.2. Interestingly, in the PDL group the frequency of cells with 0.0-0.2 NAR was approx. 60 % and thus being higher than that of ED and even hAT groups, and moreover the frequency of cells with NAR 0.5-1.0 was very similar to that of NT group.

To examine whether the implanted PDL and hAT cells were integrated in the Achilles tendon, we have performed cell tracking by staining with anti-human HLA-ABC antibody (Fig. 5d). No signals were observed in NT and ED groups as expected, while in the PDL and hAT groups,

incorporated human cells surviving or being their progeny were detected suggesting direct contribution as well as long-term survival in the defect area.

Taken together, based on the cell morphometry data together with the above-described results obtained in a very clinically relevant *in vivo* model, we firmly concluded that implantation of PDL cells aids in Achilles tendon healing that is on one side comparable to hAT cells and on the other more advanced and superior to the healing of ruptured non-implanted tendons.

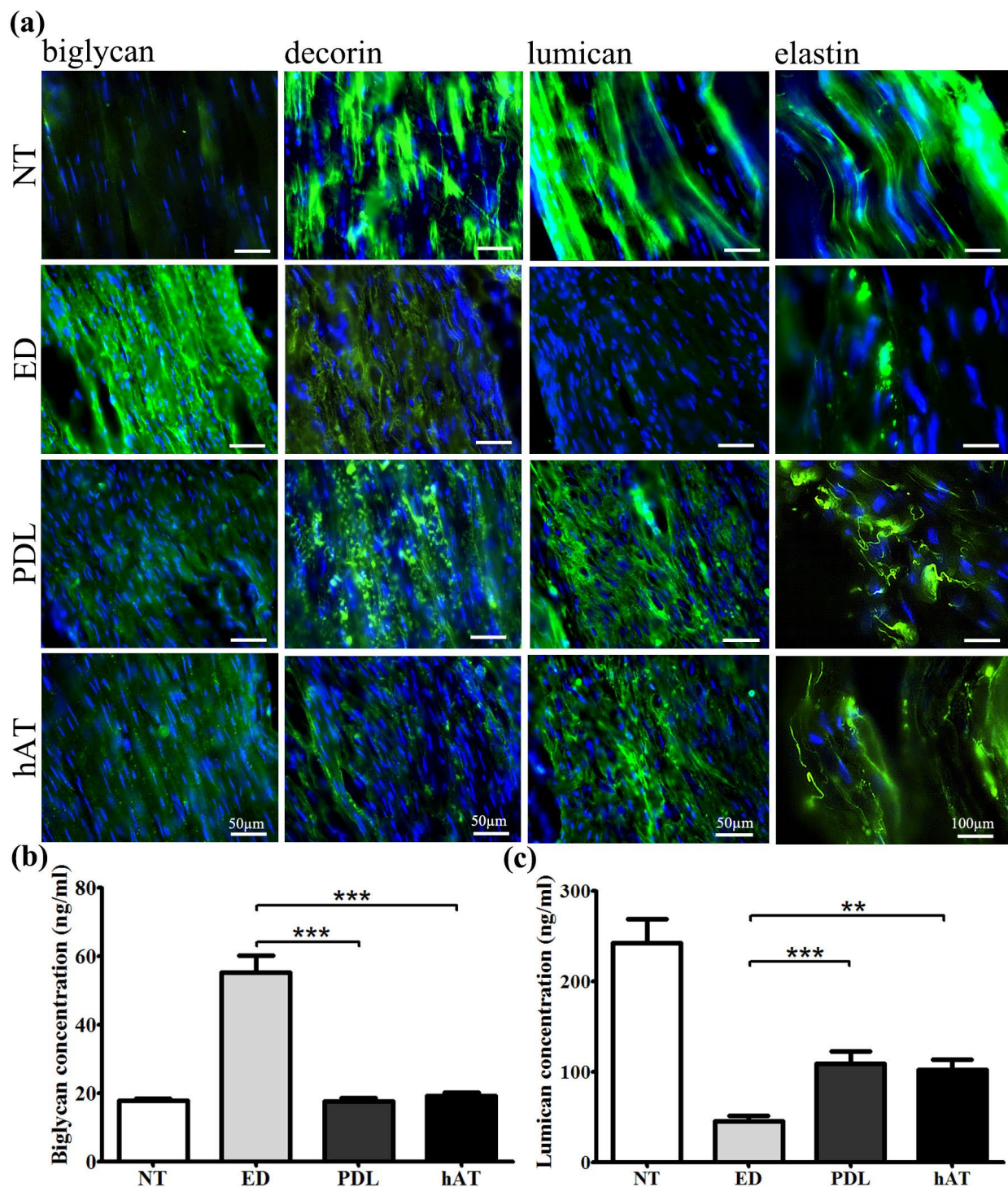


Fig. 4. Expression of key proteoglycans and elastin fibres. **(a)** Representative immunofluorescent images of the tendon-related matrix proteins biglycan, decorin, lumican and elastin (all in green colour) and nuclear counterstain DAPI (in blue) expressed in the Achilles tendons of the four study groups, NT, ED, PDL and hAT. All staining were reproduced thrice independently, including sections from 3 to 5 animals/group. **(b)** Biglycan and **(c)** lumican protein levels quantified by ELISA. Bar charts present mean \pm standard deviation; *** $p < 0.005$, ** $p < 0.01$.

Discussion

The frequent failure of fully satisfying tendon repair following age-related degeneration and overuse tendon ruptures has encouraged the search for novel therapeutic approaches to enhance the healing process of tendons. A major theme in the ongoing strategies is in the application of stem cells in injured tendons (Lui and Ng, 2013; Oragui

et al., 2012; Siddiqui *et al.*, 2010). Stem cells can be simply defined as a cellular population with the ability to self-replicate through mitosis to form daughter cell lines, which have the potential to terminally differentiate into a number of different cell lineages depending on the signalling milieu reaching these cells. In recent years, several tissues such as bone marrow, fat or tendons themselves have been identified as potential sources for obtaining stem cells for

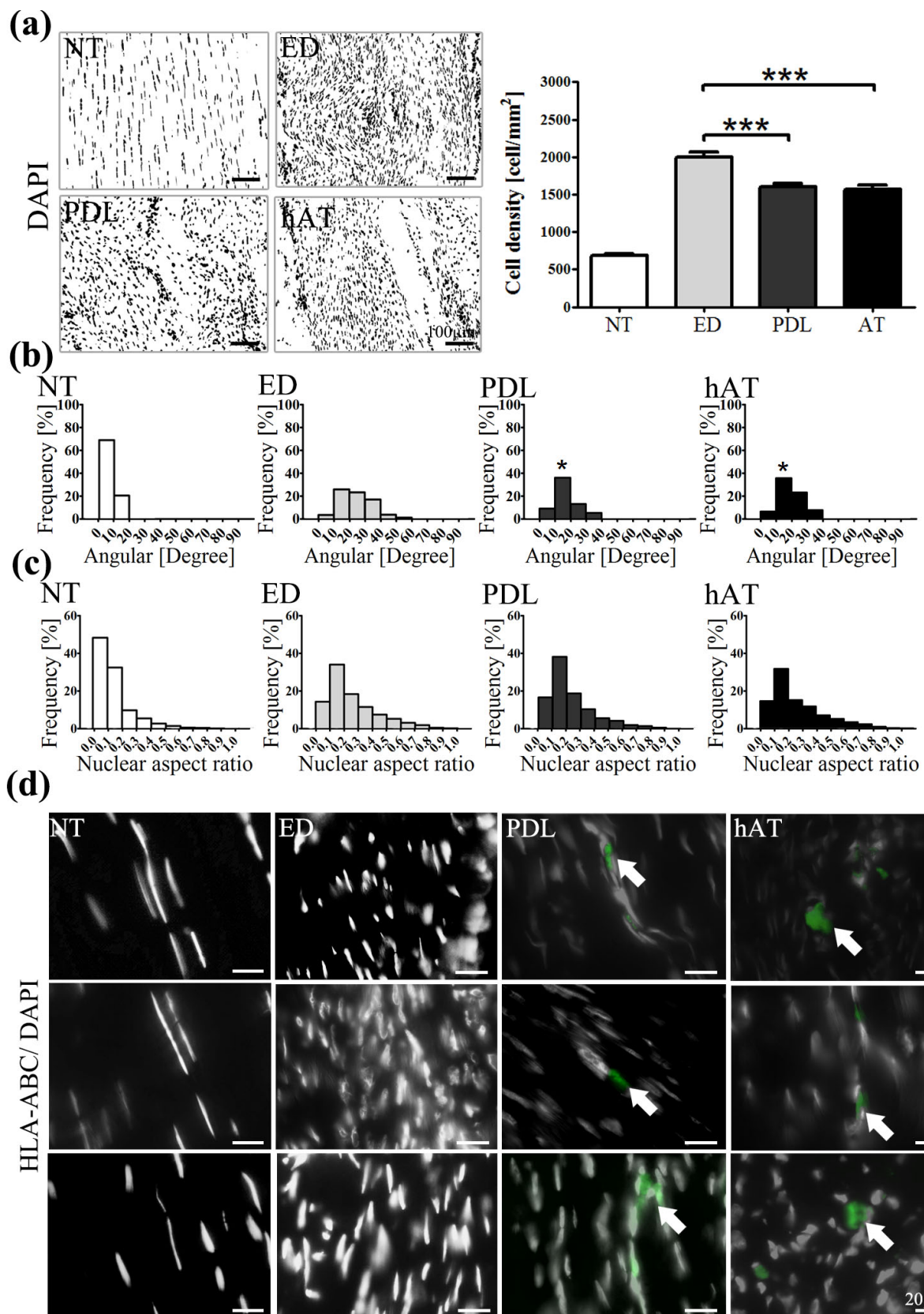


Fig. 5. Analyses of cell density, angular deviation, nuclear aspect ratio and *in vivo* tracking of the implanted human cells. **(a)** Representative black and white-converted images of DAPI staining and chart of calculated cell density. Bar charts present mean \pm standard deviation; *** $p < 0.0001$. **(b)** Frequency distribution of angular deviation (angle between individual nuclear axis and tendon longitudinal axis); * $p = 0.01$ for PDL vs. ED and * $p = 0.02$ for hAT vs. ED for the frequency of 10-20 deviation. **(c)** Frequency distribution of nuclear aspect ratio (NAR, width vs. length of nucleus) in the four study groups, NT, ED, PDL and hAT. **(d)** Immunostaining for the human-specific antigen HLA-ABC. Representative images for three different animals per group where in positively stained human cells are in green and the nuclear counterstain DAPI in white.

tendon repair. As mentioned in the introduction, up-to-date, each of these tissues faces several inevitable challenges. With regards to native tendon-derived cells, a clear advantage is that they can differentiate more efficiently directly into tenocytes rather than cells of other lineages. In our previous study (Pietschmann *et al.*, 2013), where we established the clinically relevant full-size Achilles tendon defect model, we compared the healing potential of rat MSC *versus* rat-derived tenocytes. Based on our histological and biomechanical results demonstrating reduced central ossification, increased tendon-like areas in the site of injury and above all significantly improved ratio of failure strength to tendon cross-section, we strongly concluded that tendon-derived cells hold a clear advantage to MSC. However, one of the most critical issues with the use of tendon cells is the practical limitation behind their obtainment in clinical scenarios. Because of the much easier isolation procedure of periodontal ligament, which can be simply collected from removed teeth during usual orthodontic procedures, as well as the much comparable cellular features of ligamentous and tendinous cells, we hypothesised that PDL could provide a uniquely well-suited cell source for tendon repair. Hence, in this study we utilised, the previously established by us and evaluated, the healing impact of PDL-derived cell line *versus* control Achilles tendon-derived cell line (hAT). The main advantages of using cell lines in our experimental model are: being well-characterised, allowing standardisation of the methods and expansion to high cell numbers. However, as discussed below such cell lines are only the first steps in establishing a potent clinical translation protocol because they cannot represent donor variability and be used in patients.

The two study groups, PDL and hAT were compared to non-implanted, but surgically ruptured Achilles tendons (ED group) at 16 weeks postoperatively. This time point corresponded to the late remodelling phase of the tendon healing course wherein major molecular events affecting matrix maturation, cellular organisation and transforming the initial fibrous template to the tissue pattern of healthy tendon are taking place. Based on the combination of tissue and cell morphometric, polarised light microscopic, various histological and immunohistological analyses we found that the PDL group is characterised by smaller tendon diameter, better tissue morphology, advanced matrix maturation and cellular organisation compared to the ED group. This study group was very similar to the control hAT group and both together resembled in several of the studied parameters the NT group. Moreover, *in vivo* cell tracking demonstrated that the implanted human cells or their progeny can integrate and survive long-term at the site of injury suggesting direct contribution to the healing process. In sum, we can report that PDL-derived cell implantation results in advanced and superior remodelling stage of tendon healing and can be foreseen as a novel alternative to tendon cell source to treat tendon injuries.

The implemented cell lines in our study serve as a great experimental model because they allowed standardised investigation to validate our hypothesis that PDL-derived cells are suitable source for tendon healing. This first step on selecting a high potential cell source in clinically

relevant full-size Achilles tendon defect in a small animal model provides the translational bases for designing a true clinical application. The second step should focus on scaling up to comparable tissue dimensions in big animal model such as pig, and using autologous derived PDL cells. Finally, to further speed up the healing, a possible combination of PDL cells with suitable biomaterial carrier should be considered. In such future studies in-depth histological assessment should be combined with key biomechanical measurements and functional performance tests with long-term follow up.

Our novel findings are in line with a recent study by Moshaverinia *et al.* (2014), who investigated the capacity of two dental tissue-derived cells, periodontal ligament stem cells (PDLSCs) and gingival mesenchymal stem cells (GMSCs) to form tendon-like structures *in vivo*. The two different cell types were transplanted subcutaneously into mouse dorsal surfaces and the ectopic neo-tendon regeneration was evaluated by qPCR measurements for biglycan, decorin and tenomodulin after 4 weeks and then after 8 weeks by histological and immunohistochemical stainings for tendons-specific protein markers. The authors concluded that PDL as well as GM stem cells can contribute to tendon-like formation *in vivo*. In this respect, our study presents the next progress step in assessing the healing potential of PDL cells since it demonstrates for the first time that they can indeed enhance the repair of full-size tendon ruptures in a manner that is very comparable to native tendon-derived cells.

Similar to PDL cells, researchers have dealt with other cell types that also have easier accessibility; for example, MSC from human bursa tissue that can be obtained from patients undergoing rotator cuff surgery (Song *et al.*, 2014) and induced pluripotent stem cells (Xu *et al.*, 2013) that can be generated by genetic modifications and banked in high numbers. Moreover, these studies have demonstrated that these cells exhibit a high potential for application in tendon repair. However, at present their use is associated with low efficiency of *in vitro* expansion; issues with allogeneic transplantation; phenotype stability and differentiation control. PDL is a novel attractive cell source because it can be obtained from exfoliated deciduous teeth of children aged 5 onwards and from sound teeth that are normally discarded in frequent routine orthodontic procedures (premolars in class II malocclusion or wisdom teeth removal) in dental clinics. Patient-specific PDL-derived cells can be easily harvested by scratching the surface of the root and *in vitro* pre-expanded to high numbers in culture with simple standard procedures. Possible bacterial, fungal or mycoplasma contamination can be easily controlled by using antibiotics, antifungal agents or elimination kits. Harvested PDL cells can thereafter be banked up for years ahead and last the phenotype of the cells is very similar in nature to tendon cells, hence no complicated genetic or growth factor manipulations are required. Therefore, the PDL can be considered an ideal source for stem cell banking purposes and be used in therapy of tendon injuries. Several open questions remain to be clarified in following investigations such as the possible combination of PDL cells with suitable biomaterials; the scaling up to dimensions similar to human tendon injuries; the reached

biomechanical strength; the risk of PDL cell quality loss after long-term storage; the exact technique and time point of application; and the prognosis on periods for patient hospitalisation and post-operative physiotherapy. It will be of great interest, by using adequate animal models, to examine whether PDL-derived cells are also suitable for prevention or therapy of tendon/ligament degenerative diseases, that are occurring prior rupture, and for periodontitis. We believe that further research on these questions will be critical to translating our animal model to potential clinical applications.

Conclusions

In this study, the effect of PDL for cell-based therapy of tendon injuries was investigated using an *in vivo* model of a full-size rat Achilles tendon defect. Our in-depth results demonstrated that PDL cell-implanted tendons exhibited not only advanced tissue maturation, less ectopic bone formation, more organised collagen fibres, tendon matrix expression corresponding to the final healing stage and better cell-morphometry parameters when compared with the ED group, but were also very similar to the tendons treated with hAT-derived cells. Taken together, our study clearly demonstrates the feasibility of using PDL cells as a novel cell source for tendon repair and strongly recommends this cell type for the future development of innovative regenerative applications for treatment of different tendon or ligament pathologies.

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Discussion with Reviewer

Christopher Niyibizi: For future clinical application, PDL cells would be harvested from different individuals, is there a worry for potential immune response to the donor cells by the recipients?

Authors: We foresee the use of PDL cells in two applicative modes: autologous or allogeneic. The autologous PDL cell implantation eliminates problems with immune rejection of the grafted material. In cases of low harvest of PDL cells allogeneic PDL cell implantation comes in question.

Interestingly, two studies have already demonstrated that PDL-derived stem/progenitor cells possess low immunogenicity and actually can play immunomodulatory roles. Ding *et al.* (2010), observed no immune rejection of allogenic PDL cells transplanted in a swine model of periodontitis. Kim *et al.* (2010) showed that human and canine PDL cells have profound immunomodulatory effect (inhibition of cell division) on human peripheral blood mononuclear cells. The above results suggest that PDL donor cells can circumvent immune rejection; however, follow up xenogenic *in vivo* studies are necessary to strengthen such findings prior clinical testing.

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